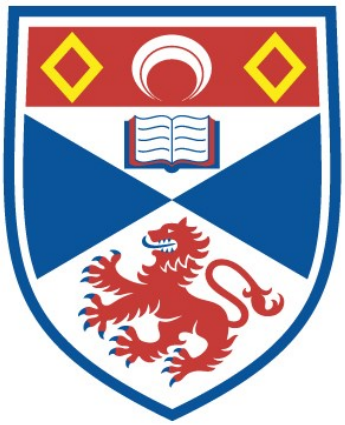
SOME ASPECTS OF THE NEUROBIOLOGY OF OPHIUROIDS : WITH SPECIAL REFERENCE TO OPHIURA TEXTURATA (L.) (ECHINODERMATA, OPHIUROIDEA)

Timothy Richard Stubbs

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1983

ABSTRACT

Some Aspects of the Neurobiology of Ophiuroids with Special Reference to Ophiura texturata (L.) (Echinodermata, Ophiuroidea).

By

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A study has been made of the anatomy and physiology of the nervous system of Ophiura texturata, involving the use of light and electron microscopy and of extracellular and intracellular electrophysiological recording techniques. A previously unknown ciliary feeding structure in O.texturata has also been described.

The nerve cords of O.texturata contain a system of large neurones that are an order of magnitude larger than the nerve cells occurring in members of other echinoderm classes. These large neurones have been designated as "giant" neurones. The size of the giant neurones has made it possible to trace their extent within the nerve cords and thus to produce the first detailed description of the cellular structure of an echinoderm nervous system. The radial nerve cords consist of a chain of structurally similar segments, and degeneration studies have shown that each segment contains a separate population of neurones. The circumoral nerve ring which has previously been regarded as the controlling centre

of the echinoderm nervous system, has a structure which is consistent only with providing a functional connection between adjacent radii and not with the task of central integration.

The electrical activity of single units within the ectoneural part of the nerve cords of O.texturata has been recorded in response to a variety of stimuli, by the use of extracellular suction electrodes. Decrementally conducted compound potentials, reported in previous electrophysiological studies of echinoderm nervous systems, were not recorded. Intracellular recordings of the activity within single giant cells have also been obtained. This is the first report of intracellularly recorded nervous activity in any echinoderm. The intracellular work is of a preliminary nature and suggestions for further study are made.

The genital shields in O.texturata are covered by an array of ciliated ridges and non-ciliated grooves. An examination of this structure indicates that it is specialized for a form of ciliary/mucus suspension feeding. These structures also provide a specialized preparation for the study of some aspects of the function of the sub-epidermal nerves in echinoderms.

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SOME ASPECTS OF THE NEUROBIOLOGY OF OPHIUROIDS WITH SPECIAL REFERENCE TO *Ophiura texturata* (L.) (ECHINODERMATA, OPHIUROIDEA).

BY

TIMOTHY RICHARD STUBBS

A thesis presented for the degree of Doctor of Philosophy at the University of St. Andrews

Gatty Marine Laboratory

University of St. Andrews

September 1982

SUPERVISOR'S CERTIFICATE

I certify that T.R. Stubbs has fulfilled the conditions laid down under Ordinance General No. 12 of the University of St. Andrews and is accordingly qualified to submit this thesis for the degree of Doctor of Philosophy.

DECLARATION

I declare that the work reported in this thesis is my own and has not previously been submitted for any other higher degree.

VITAE

I was educated at Kings School Bruton, Somerset and the University of St. Andrews where I graduated in Zoology in 1979. The work described in this thesis was carried out between October, 1979 and August, 1982.

ACKNOWLEDGEMENTS

I thank Dr. J.L.S. Cobb, under whose supervision this work was carried out, for his guidance, tolerance and enthusiasm.

Thanks are also due to all the staff and students of the Gatty Marine Laboratory for their advice and helpful discussion on many occasions. In particular thanks are due to Dr. W.J. Heitler and Mr. Keith Sillar for their helpful suggestions about intracellular technique.

The work described in this thesis was carried out during the tenure of Science and Engineering Research Council Studentship No. B/79305546.

Note: Ophiura texturata has been renamed Ophiura ophiura. See Clark, A.M. (1976). Bull. Zool. Nomenclature 32(4).

CONTENTS

GENERAL INTRODUCTION

Echinoderms are fundamentally radially symmetrical animals, and in all but a few species they exhibit, to some degree, a pentameric body form. The lack of a head and of any degree of cephalization has resulted in a form in which the mouth is located centrally and is often closely applied to the substratum, or points vertically upward. As an indirect consequence of this arrangement the nervous system is circumorally disposed, with extensions into the arms or ambulacra. The nervous system is structurally a relatively simple system with much of it retaining a position close to the epidermis. Although a brain is lacking, some concentration of the nervous elements has occurred resulting in the formation of the radial nerve cords and circumoral nerve ring.

The classical histological studies on which much of our present knowledge of the structure of the echinoderm nervous system is based, were carried out in the latter part of the nineteenth century, and have since been extensively reviewed by Hyman (1955). In the same period a number of simple behavioural experiments on echinoderms were also reported (von Uexkull, 1896). Echinoderms have attracted attention from a number of workers since that time, and most of this work has also involved studies of behaviour (see Reese, 1966 for a review). Anatomical studies by Smith (1937a, 1947, 1950a), Kawaguti (1965), Kawaguti et al. (1965) and Cobb (1967a&b; 1968a,b&c) have confirmed the basic findings of earlier work and have added a great volume of new and important information about the fine structure of the echinoderm nervous

system. Studies of the fine structure of asteroid and echinoid radial nerve cords led Cobb (1970) to propose a theoretical model of the echinoderm nervous system. This was the first attempt since Smith's work to correlate the structure of the nervous system with function. The earliest reported studies which include work on the physiology of the echinoderm nervous system were concerned with the contraction of holothuroid muscles (Tao, 1927; Buddington, 1937). Later work on nerve/muscle systems in holothuroids by Boltt and Ewer (1963a&b) and by Pople and Ewer (1954, 1955, 1958) produced some of the first models of the echinoderm nervous system based on the results of physiological experiments.

Published electrophysiological studies of echinoderms are rare and all save two, those of Takahashi (1964) and of Brehm (1977), have failed to detect activity within single nerve cells. Studies of conduction within the nerve cords of asteroids and of echinoids have found only compound potentials which have low velocities of conduction and which display a loss of amplitude with distance. The results of work on the nervous control of bioluminescence in a californian ophiuroid, Ophiopsila californica (Brehm, 1975,1977) produced the first indication of the possibilities of the ophiuroid nervous system for the study of echinoderm neurobiology; extracellular suction electrodes were used to record the electrical activity of single units within the radial nerve cords.

The study of the echinoderm nervous system presents considerable technical difficulties; many of the techniques which work well with members of other phyla fail to produce results when applied to echinoderms. Many of the difficulties stem from the extremely small size of most echinoderm neurones. Ophiuroids however possess nerve cells which are large by echinoderm standards and which are therefore more amenable to neurobiological study.

The work reported in this thesis deals with a number of aspects of the neurobiology of ophiuroids. The majority of the work was performed using Ophiura texturata, a highly active subtidal brittlestar. This report is divided in to three chapters, the first of which deals with the anatomy and ultrastructure of the nervous system of O.texturata, and provides a description of a system of large neurones found within the nerve cords of this animal. These large nerve cells are designated as "giant" neurones since they are an order of magnitude larger than the majority of echinoderm neurones. The size of the giant nerve fibres has made it possible to trace their extent within the nerve cords, and thus to produce the first detailed description of the cellular layout of an echinoderm nervous system. The results of the anatomical studies described in the first chapter provide a basis for the interpretation of the physiology. Electrophysiological work is described in the second chapter, and much of this is concerned with single unit activity evoked by photic stimulation. Intracellular recordings have been made from single ophiuroid giant neurones and

these are the first such recordings reported in any echinoderm. The intracellular work is preliminary in nature and provides a basis for further study. The third chapter consists of a report of previously undescribed ciliary feeding structure in O.texturata; the interest in these structures is not merely in relation to feeding mechanisms in ophiuroids, but they also provide a specialized preparation for the study of some aspects of the function of the sub-epidermal nerves in echinoderms.

Collection and Maintainance of Specimens

Ophiura texturata (Fig. 1) is a sub-tidal brittlestar which occurs extensively in the Atlantic, North Sea, English Channel, Baltic and Mediterranean. It may be found from the low tide line to depths of up to 200 metres. Specimens may have disc sizes between two or three millimetres and three centimetres in diameter. Small specimens for this study were collected from the St. Andrews Bay area with the aid of a beam trawl. Larger specimens (with disc diameters exceeding 1.5 cm) were supplied by the Millport Marine Station on the Isle of Cumbrae. The animals were kept in sand-bottomed circulating seawater tanks; the water temperature and light and dark cycles were not strictly controlled.

Specimens of other species of brittlestar used for comparative purposes in this study were obtained as follows: Ophiothrix fragilis (Fig. 2) was collected from rock pools in the St. Andrews Bay area, and Ophiocomina nigra (Fig. 3) was collected off the

Fig. 1

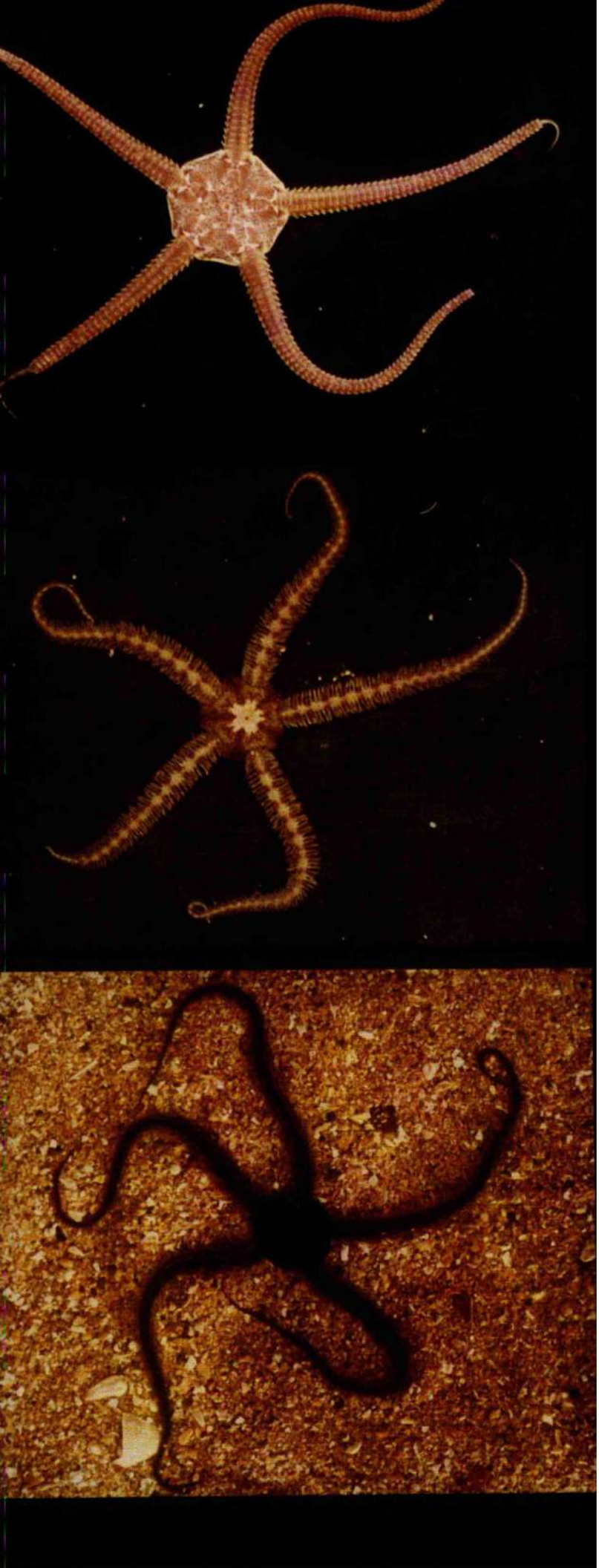
Ophiura texturata

Fig. 2

Ophiothrix fragilis

Fig. 3

Ophiocomina nigra



Isle of May in the Firth of Forth, by divers. Specimens of both species were maintained under similar conditions to those described for O.texturata.

CHAPTER 1

ANATOMY

INTRODUCTION

There have been few published accounts of the structure of the ophiuroid nervous system since the classical studies carried out in the late nineteenth century by Christo-Apostolides (1882), Hamman (1889), Cuenot (1891) and von Uexkull (1896). The only two recent neurohistological studies of ophiuroid tissue have involved light and electron microscopic investigations of the radial nerve cords of Ophiopsila californica (Brehm,1975) and of the hyponeural tissue of the radial nerves of Ophiothrix fragilis (Pentreath and Cottrell, 1971). However, there are a number of published ultrastructural studies of the radial nerves of asteroids (Kawaguti,1965; Cobb,1970; Cottrell and Pentreath,1970) and of echinoids (Kawaguti et al.,1965; Cobb,1970). These studies revealed that the bulk of the neurones making up the radial nerves of both starfish and sea urchins are small. Rarely does the cell body exceed 5 um in diameter at its widest point and axon diameters range between 0.1 um and 3 um.

The majority of ophiuroid neurones are similar in size to those of echinoids and asteroids, however some are much larger: Brehm (1975) described ectoneural axons in the radial nerve cords of Ophiopsila californica that are up to 8 um in diameter, and

Pentreath and Cottrell found that some of the hyponeural motor axons of Ophiothrix fragilis reached 10 um in diameter. The only other report of the occurrence of large axons in echinoderms was by Christo-Apostolides (1882) who reported the presence of two "giant" fibres within the radial nerve cords of Ophioglypha.

The ultrastructural studies of the nervous system of Ophiura texturata described in this chapter demonstrate that this species possesses an extensive system of "giant" fibres within the ectoneural tissue of both the radial nerves and the circumoral nerve ring. The hyponeural tissue in O.texturata is composed exclusively of neurones that are giant by echinoderm standards.

The structure of the circumoral ring is also described in this chapter. No account of the structure of the circumoral nerve ring in any echinoderm has been published since the late nineteenth century. The circumoral nerve ring has been regarded by many as the "brain" of an echinoderm. This idea was proposed to account for the results of numerous behavioural experiments which suggested the presence of controlling centres within the nervous system. The behavioural evidence was reviewed by Reese (1966) and little further work on the subject has been reported since that time. Considering the assumed importance of the circumoral ring it is surprising that no detailed report of its structure has yet been published.

Many of the models of circumoral nerve ring structure proposed from the results of behavioural work are not consistent with the ultrastructural evidence reported in this chapter. Smith (1965) predicted that the nerve ring would have a complex structure, "one may suppose with some confidence that the nerve ring will be found to have a more complex structure than the cord": the ultrastructural evidence demonstrates quite the reverse. Smith's supposition that giant fibres would be found within the nerve cords of brittlestars is however borne out by this study, and by the work of Brehm (1975) and Pentreath and Cottrell (1971).

This chapter also provides a detailed description of the innervation of the the musculature of the arms by the hyponeural motor nerves.

MATERIALS AND METHODS

The structure of the nervous system of Ophiura texturata was investigated using both light and electron microscopy. Degeneration studies were used to trace the extent of individual fibres within the nerve cords. Attempts to investigate the morphology of single neurones using Golgi preparations and cobalt backfilling techniques were also made.

Microscopy

The problems associated with the fixation of marine invertebrate, and particularly of echinoderm tissue have received considerable attention (see Baur and Stacey,1977; Cobb and Mullins, 1973; Cobb and Pentreath, 1978; Eisenman and Alfert,1982) and a variety of fixation methods were investigated in the course of this study in an attempt to achieve satisfactory standards of preservation. These methods included those of Baur and Stacey (1977), Bachmann et al. (1980), Coleman (1969), Eisenman and Alfert (1982), Holland and Nealson (1978), Hylander and Summers (1975) Prosser and Mackie (1980) and Wood and Cavey (1981) (see Appendix A for details of methods). Most of these techniques employed gluteraldehyde fixation followed by post fixation in osmium tetroxide in a variety of buffers (s-collidine, cacodylate, phosphate and PIPES). The following method was eventually chosen as the one yielding the most satisfactory results with all the tissues studied.

Specimens were initially anaesthetized in a 1:1 mixture of 0.36M magnesium chloride and seawater for between five and ten minutes. Pieces of tissue, as small as practicable, were fixed in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) for one hour at room temperature. The fixed tissue was rinsed in three changes of 0.3M sodium chloride for five minutes per change and was subsequently decalcified in a 1:1 mixture of 2% ascorbic acid and 0.3M sodium chloride for 24 to 48 hours (modified from Dietrich and Fontaine, 1975). Decalcified tissue was rinsed in distilled water, dehydrated in acetone and embedded in either Durcupan or Spurr's resin (Spurr,1969). In cases in which isolated nerve cords were processed for microscopy the decalcification stage was omitted.

Thick (1 µm) sections were stained with toluidene blue and examined with the light microscope. Ultrathin sections were stained with uranyl acetate and lead citrate and were examined using an AEI EM6B operated at 60 KV.

The structure of the radial nerve cords and the circumoral nerve ring and their peripheral branches were investigated using sets of serial 1 µm sections cut in transverse, longitudinal and horizontal planes and examined with the light microscope. The ultrastructure of selected areas was examined in further detail using an electron microscope.

Golgi Studies

The technique used for these experiments was based on the rapid Golgi method of Butler (1971). Golgi impregnations of invertebrate material are notoriously difficult but Keenan et al. (1981) successfully used a modification of the Butler technique to study the morphology of single neurones in the brain of the polyclad flatworm Notoplana acticola, and they suggested that their technique might be of use in the study of other invertebrate nervous tissue. Keenan's modification of the Butler technique was thus used for this study.

Sections of radial nerve cord with parts of the circumoral nerve ring attached were dissected out from anaesthetized brittlestars and immediately fixed (see Table 1), and were then placed in the dark for 4 hours. The fixed nerve cords were removed from the fixative using glass or wooden spatulas and following a brief rinse in distilled water were placed in the post fixative, in the dark, for 12 hours. On completion of post fixation the tissue was rinsed several times in in 0.75% silver nitrate and subsequently placed in the dark in fresh 0.75% silver nitrate for 12 hours. The timings for individual stages were those found to be optimum by Keenan et al. (1981). Variations in the timing of each stage (see Table 1) were introduced in an effort to achieve successful impregnation of the tissue. All the stages were carried out at room temperature. Impregnation was followed by dehydration in an ethanol series and clearing in methyl salicylate. Whole mounts were examined with the light microscope.

Process	Reagents	Procedure	Time
Fixation	6% glutaraldehyde Millonig's phosphate buffer (pH 7.4) Sucrose	Mix 1 part glutaraldehyde in 1 part of Millonig's buffer. Add sucrose to make a 0.45 M solution.	3-12 hrs
Post-fixation	2.5% potassium dichromate in 6% glutaraldehyde Sucrose	Add sucrose to make a 0.45 M solution. Place tissue in the solution in the dark.	8-24 hrs
Impregnation	0.75% silver nitrate 2% OsO_4 in double distilled water	Rinse tissue in $AgNO_3$ until no further precipitate forms. Place tissue in fresh $AgNO_3$ with .01 ml OsO_4/ 10 ml $AgNO_3$. Place in dark.	12-36 hrs
Dehydration	Ascending series of ethanol	One change each to 100% ethanol. Three changes in 100%	10-20 min. each
Clearing	Methyl salicylate or xylenes	Two changes	10 min each
Mounting	Permount or Entallan		

Table 1. Reagents for rapid Golgi impregnation of invertebrate nerve tissue. Modified from Butler (1971) and Keenan et al. (1981)

Cobalt Backfilling

Attempts were made to investigate the morphology of individual giant neurones by introducing a solution of cobalt chloride via the cut ends of axons, and subsequently visualizing the stain by adding ammonium sulphide which forms a black precipitate of cobalt sulphide with the cobalt, and thus renders the cell visible under the light microscope (technique modified from Pitman et al., 1973). Backfilling experiments were attempted using both radial nerve cords and circumoral nerve rings . The nerve cords were placed on a square of plastic film pinned to the bottom of a petri dish lined with Sylgard. A small well of vaseline was constructed around the cut end of the nerve and was filled with 250 mM cobalt chloride. The well was covered over with more vaseline to prevent leakage of the cobalt chloride solution, and the petri dish filled with chilled, filtered seawater. The preparation was incubated for 12-24 hours at 4 ^{o}C. After incubation the nerve cords were transferred to fresh seawater to which 2 or 3 drops of ammonium sulphide were added. Visualization of the stain was followed by fixation in 4% formalin, dehydration in acetone and clearing in methyl salicylate. Preparations were subsequently examined with the light microscope.

RESULTS

GENERAL MORPHOLOGY OF THE NERVOUS SYSTEM

The bulk of the nervous tissue in brittlestars is aggregated to form the radial nerve cords and circumoral nerve ring. The radial nerve cords consist of a ribbon of tissue that runs along the ambulacral grooves on the oral side of each arm (Fig. 1); the nerve cord is protected from the external environment by the ventral arm plates. The radial nerve cords are swollen in each arm segment to form a ganglion and a number of nerve branches are given off at this point (Fig. 2). In common with other eleutherozoan echinoderms the nerve cords are composed of two distinct tissues, an extensive ectoneural system lies oral to the more localized hyponeural system (Figs. 2 and 3). The former is thought to contain both motor and sensory fibres whereas the latter is presumed to be entirely motor (see Hyman,1955).

The radial nerve cords unite within the disc to form the circumoral nerve ring. Examination of sets of thick (1 μm) toluidene blue-stained serial sections with the light microscope revealed the general layout of the nervous system. The radial nerve cords consist of a chain of interconnected ganglia, with one ganglion situated in each segment of the arm (the distance between adjacent intervertebral ossicles). The ganglia contain extensive areas of neuropil whilst the interganglionic regions consist of tracts of fibres aligned in parallel. Glial tissue is not present in echinoderms and this has been documented and discussed in detail by Radojcic and Pentreath (1979).

Fig. 1

Transverse section of an arm of O.texturata to show the position of the radial nerve cord (RNC).
AIM - aboral intervertebral muscle
EP - epineural sinus
OS - decalcified ossicle
T - tube foot

x 120

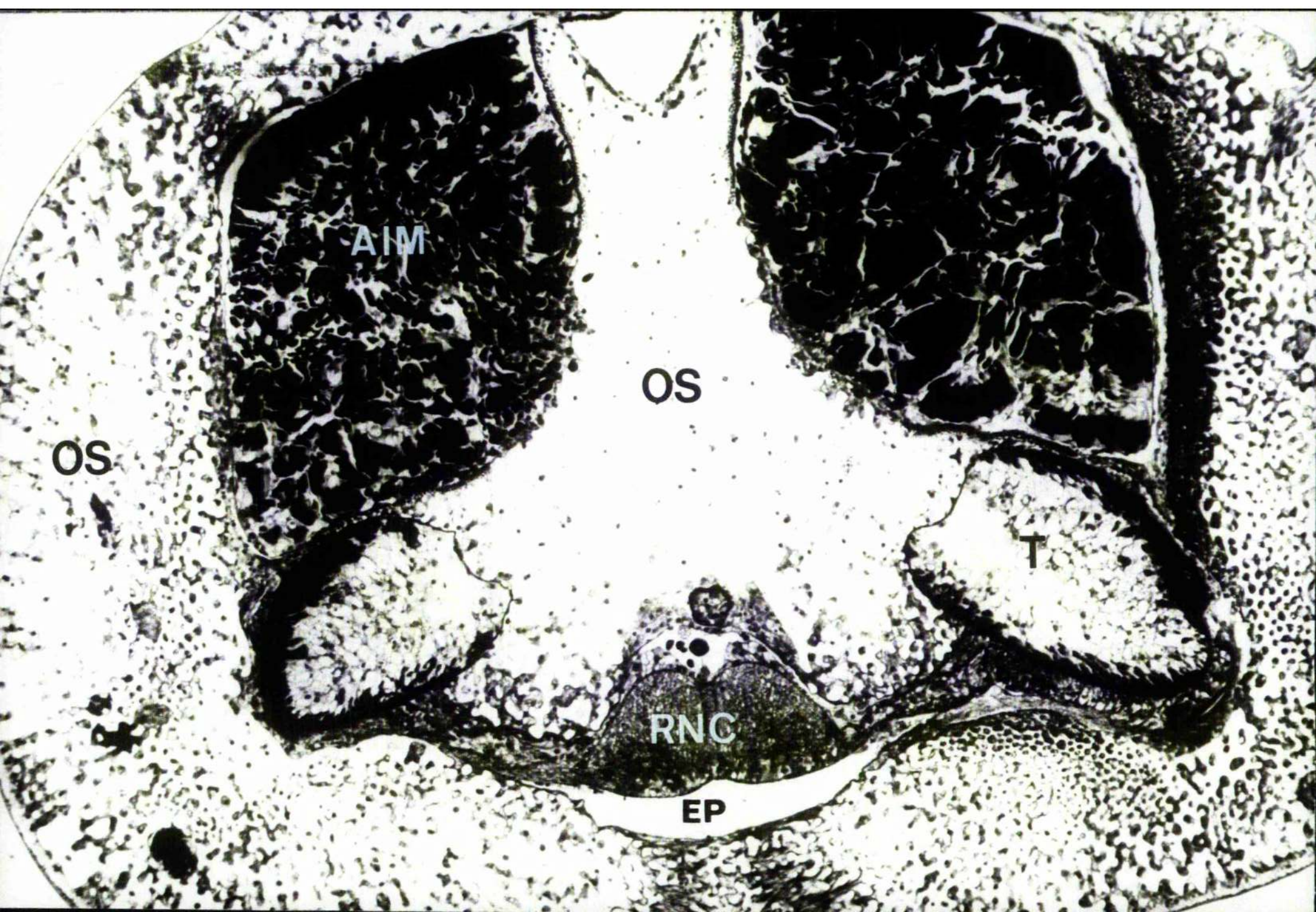

AIM
OS
OS
T
RNC
EP

Fig. 2

A diagram of the radial nerve cord of O.texturata. A number of nerve branches are given off in the region of each ganglion.

E - ectoneural tissue
H - hyponeural tissue
i - integumentary nerve
l - lateral nerve
MN - hyponeural motor nerves
p - podial nerve
r - radial haemal strand

Not to scale

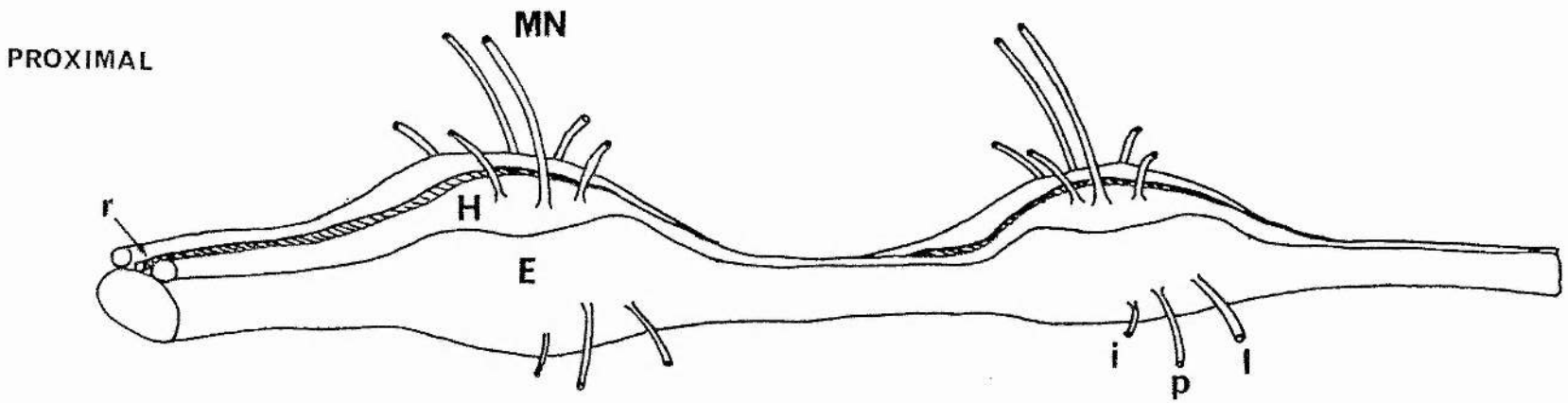
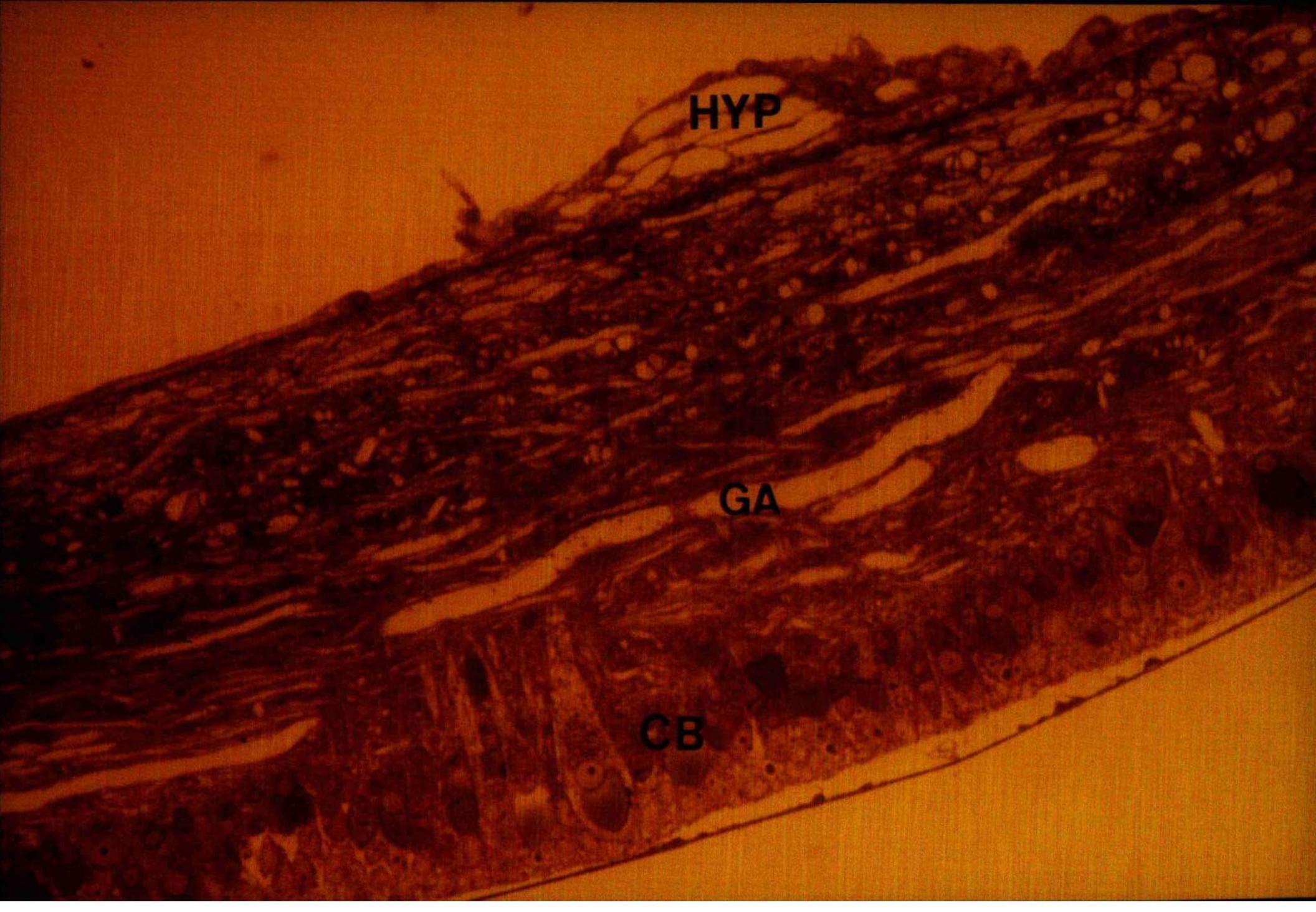

PROXIMAL
MN
r
H
E
i
p
l

Fig. 3

Longitudinal section of a radial nerve cord stained with toluidene blue. The ectoneural tissue lies oral to the hyponeural tissue (HYP). Ectoneural cell bodies (CB) are shown, as are incomplete sections of giant axons (GA).

x 350


HYP
GA
CB

Ectoneural Tissue

The oral surface of the radial nerve cord is made up of a mixture of of small epithelial cells which are between 1 μm and 4 μm in diameter and ectoneural cell bodies which range between 4 μm and 40 μm in diameter. The neurones with axons of diameters greater than 10 μm are an order of magnitude larger than any nerve cells previously described in other classes of echinoderms, and indeed than of the bulk of ophiuroid neurones. These large cells are designated as "giant" fibres. The size of the giant fibres appears to be directly related to the size of the animal. The largest axons, which can be 20 μm in diameter, are found only in the largest animals (i.e. those having disc diameters greater than 2 cm). This layer of epithelial cells and cell bodies is underlain by the epineural sinus. The epithelial cells are joined by desmosomes but they do not form a typical epithelium as they do in the Asteroidea, where there are no calcareous plates protecting the nerve cord. There are several hundred cell bodies in each segment of the nerve cord and the majority of these are at the lower end of the size range, however each segment of the nerve cord (the distance between successive ganglia) contains approximately 20 to 30 giant neurones.

Degeneration studies were used to trace the pathways of the axons of the giant neurones within the ectoneural tissue. A short section of nerve cord was removed and the experimental animal was subsequently left for between 2 days and three weeks prior to the fixation of the nerve cord. Wallerian and retrograde degeneration

took place within the nerve cord (Fig. 4) in as little as two days and much regeneration had occurred by three weeks after the initial operation. Degeneration did not take place in any of the axons in any individual beyond the first whole segment in both proximal and distal directions from the point at which the nerve cord was severed. These observations indicate that each segment of the nerve cord contains a separate population of neurones. If individual fibres ran for more than the distance between adjacent ganglia fibres showing degeneration would be found in other segments of the nerve cord.

The failure of attempts to backfill via the cut ends of giant axons was initially puzzling. However the results of the degeneration experiments made it clear that backfilling experiments using the radial nerve cord were not feasible due to the short length of individual fibres which do not run further than the distance between successive ganglia.

The cell bodies have a regular distribution occurring in four major bands in the ganglion region of each nerve cord segment (Fig. 5). The cell bodies contain a nucleus, a small quantity of rough endoplasmic reticulum and small Golgi bodies. The main volume of the cytoplasm is occupied by a structureless amorphous material (Fig. 6), which stains densely with toluidene blue (see Fig. 3). When examined with the electron microscope the cytoplasm appeared to consist of an electron dense granular material, and a proportion of the axon profiles also contained this material. In cases where material was block-stained during preparation for electron microscopy the granular appearance of the cytoplasm disappeared.

Fig. 4

Ablation of a section of the radial nerve cord results in the degeneration of the severed axons. The degenerating fibres become full of flocculent electron dense material. A degenerated axon is arrowed. Coelomocytes (CM) containing lysosomes invade the nervous tissue and eventually engulf the degenerated axons.

x13,000

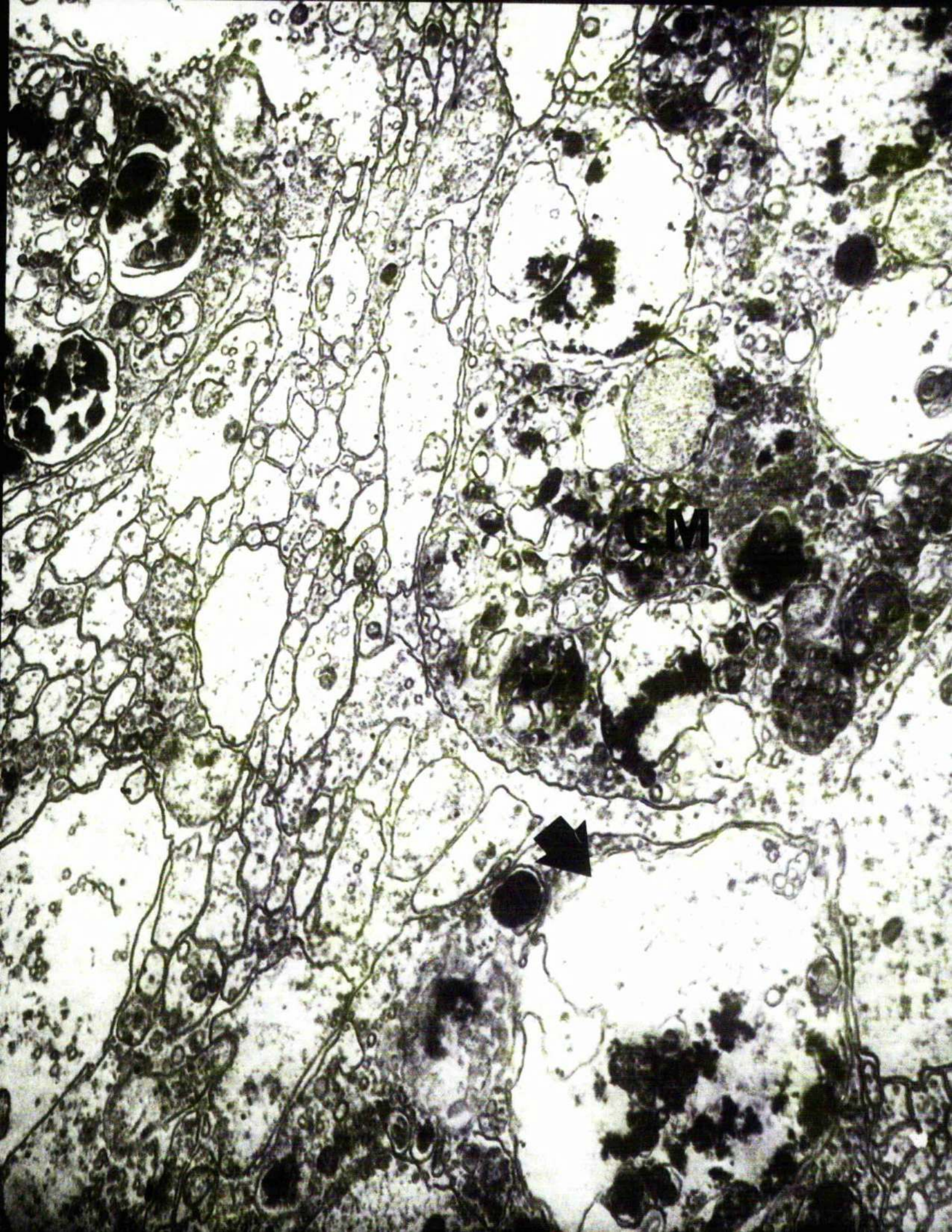

CM

Fig. 5

The radial nerve cord of O.texturata consists of a series of segmental ganglionic swellings, from which side branches are given off. This diagram is of an oral view of part of the nerve cord. There are up to 30 longitudinal giant fibres (LG) in each segment. There are symmetrically placed areas of neuropil (NP) in each segment and a precise arrangement of bundles of smaller axons such as the transverse tracts of fibres (TF). The ectoneural cell bodies cover the oral surface of the nerve cord and the largest cell bodies are arranged in clearly defined tracts in each segment. They consist of the cell bodies of monopolar and bipolar longitudinal giant axons (EN1 and EN3) and the monopolar cell bodies of vertical giant axons (EN2). There are fenestrations in the basal lamina between the hyponeural and ectoneural tissues at similar locations (FEH) in each segment.

Not to scale

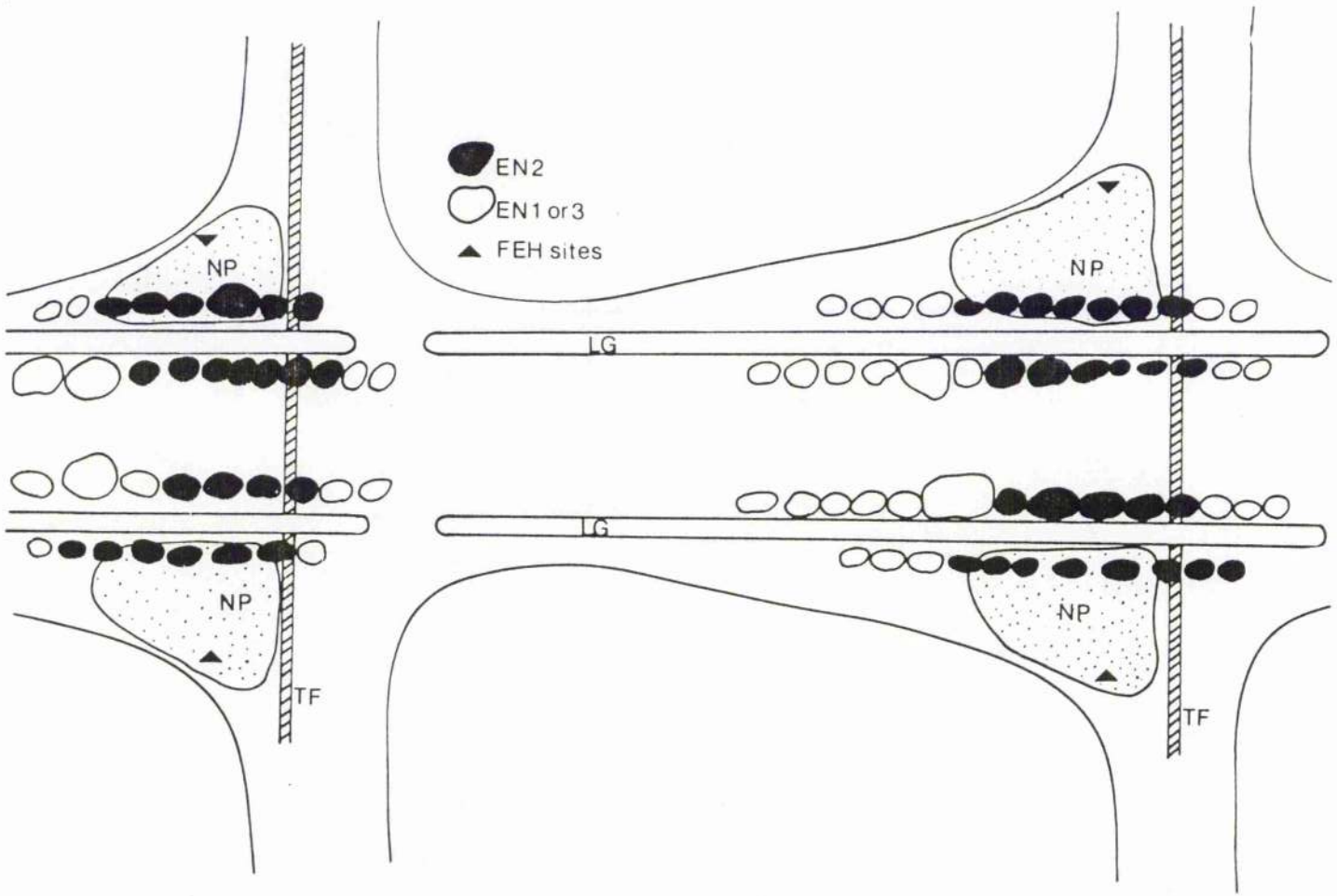
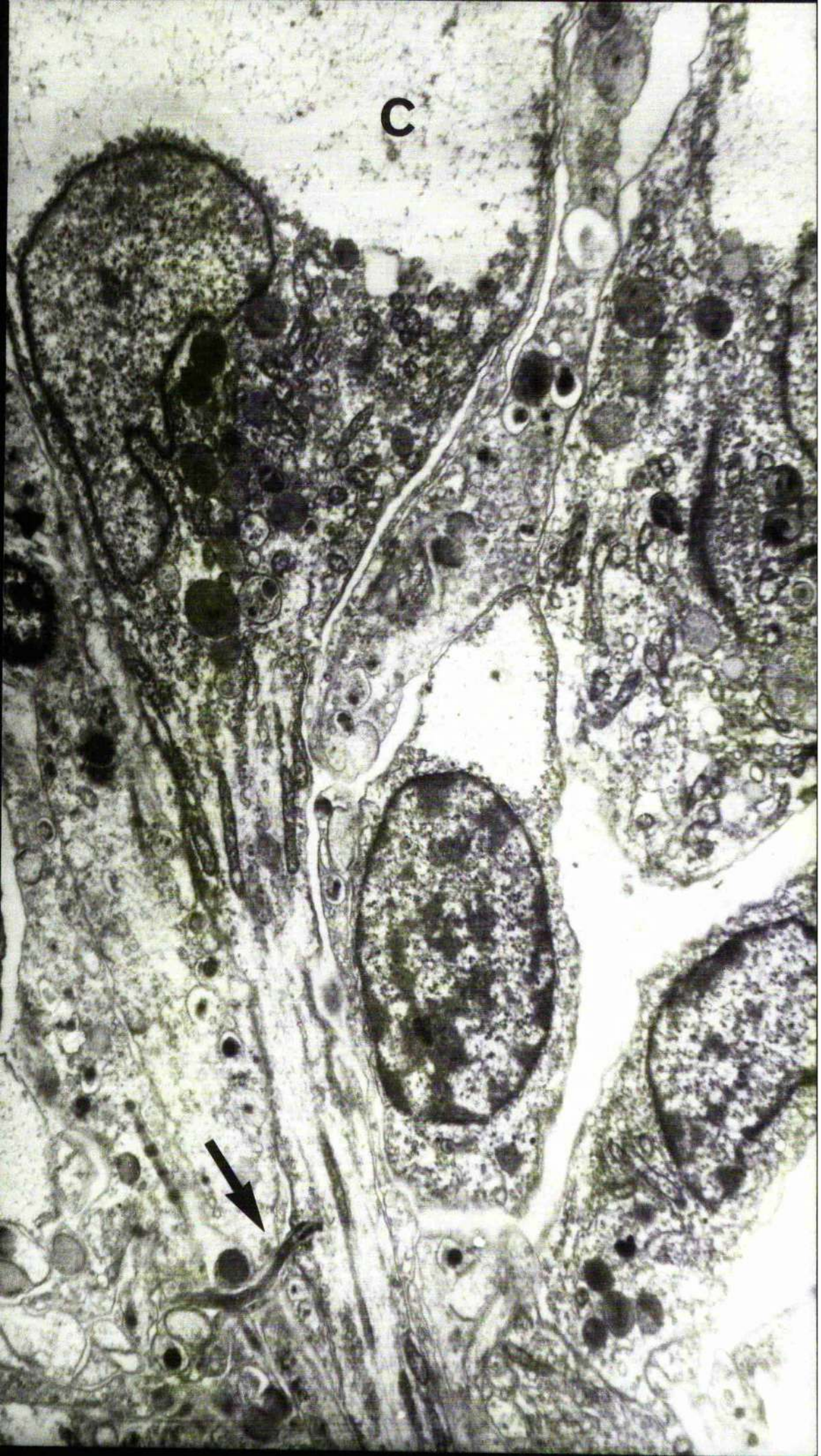

EN2
EN1 or 3
FEH sites
NP
NP
LG
LG
NP
NP
TF
TF

Fig. 6

The majority of the cytoplasm (C) of the giant cell bodies contains electron lucent material that stains strongly with toluidene blue. A modified cilium (arrow) is present in the axon hillock region.

x 7,000


C

It is possible that this granular material is glycogen although its origin is unclear.

Three major types of ectoneural giant axon can be distinguished on the basis of the path of the axon (Fig. 7). Two types have longitudinal axons up to 20 μm in diameter and are either monopolar or bipolar; the axons of the third type of giant cell rise aborally from the cell body through the ectoneural tissue and the turn through 90° to run transversely (Fig. 7). The remainder of the ectoneural tissue is made up of tightly packed small axons (averaging 1 μm in diameter) and a small number of support cells. The support cells contain 10 nm filaments (Fig. 8) and they have been previously described by Cobb (1970). In common with the giant fibres the small axons are aligned in parallel in the interganglionic regions of the nerve cord (Fig. 9), with areas of neuropil being largely confined to the ganglia.

The areas of neuropil consist of many interweaving vesicle-filled varicose axons in a random orientation. The giant fibres invade the areas of neuropil and may themselves occasionally contain vesicles.

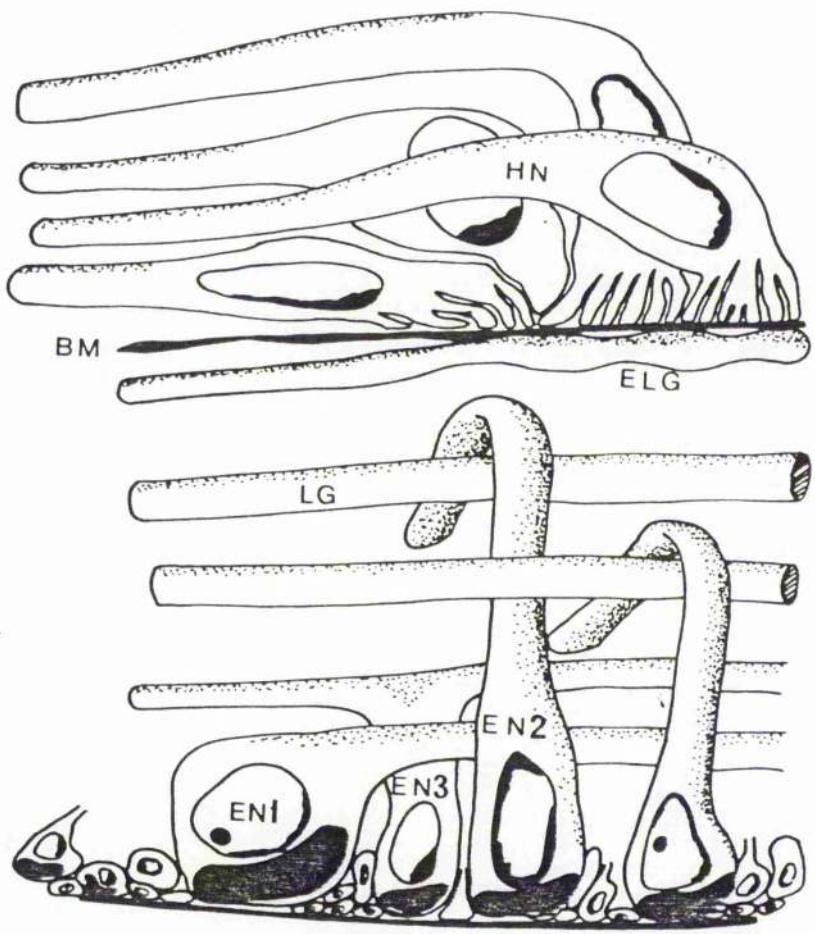
Hyponeural Tissue

The hyponeural portion of the radial nerves lies aboral to the ectoneural tissue and is separated from it by an acellular basal lamina which varies in thickness between 20 nm and 1 or 2 μm. The hyponeural tissue in each arm segment consists of a group of some

Fig. 7

Diagram of a longitudinal section through the ganglionic region of a segment of a radial nerve cord. The oral surface is covered by the aboral wall of the epineural sinus which overlies a mixture of epithelial cells (EP), normal cell bodies and giant cell bodies. The latter give rise to one of three types of axon: monopolar (EN1) and bipolar (EN3) longitudinal axons, or axons that rise vertically through the nerve cord (EN2). Distinct tracts of longitudinal fibres (LG) occur and some of the fibres form endings (ELG) at the basement membrane separating the ectoneural nerves from the plexus of small endings from the hyponeural cell bodies (HN).

Not to scale



ORAL SURFACE

Fig. 8

Small numbers of support cells (S) occur in the ectoneural tissue of the radial nerve cords and circumoral nerve ring. These cells contain a central core of 10 nm filaments. The striated appearance of the filaments in this micrograph is an artifect due to the plane of the section.

x 30,000

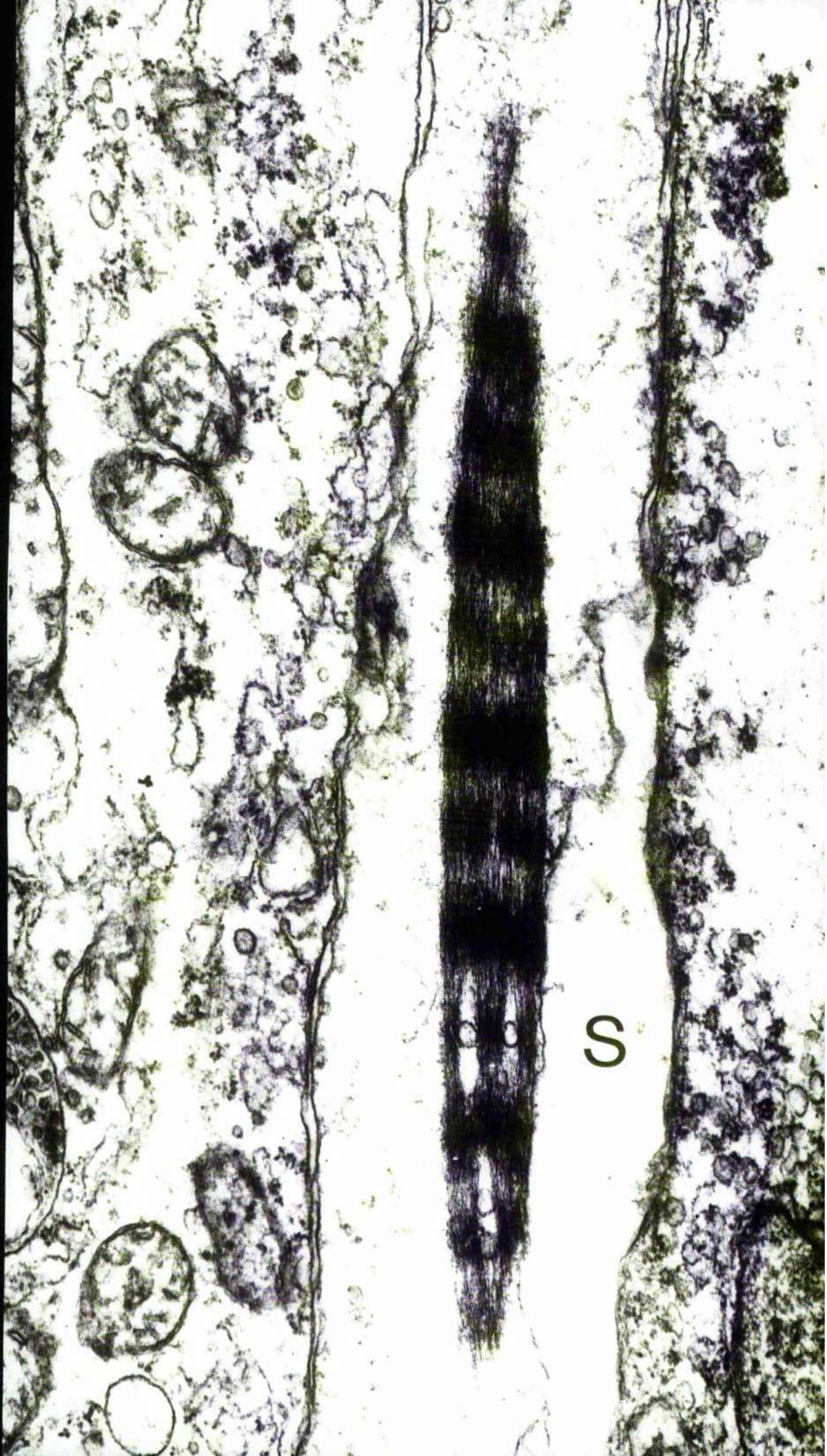
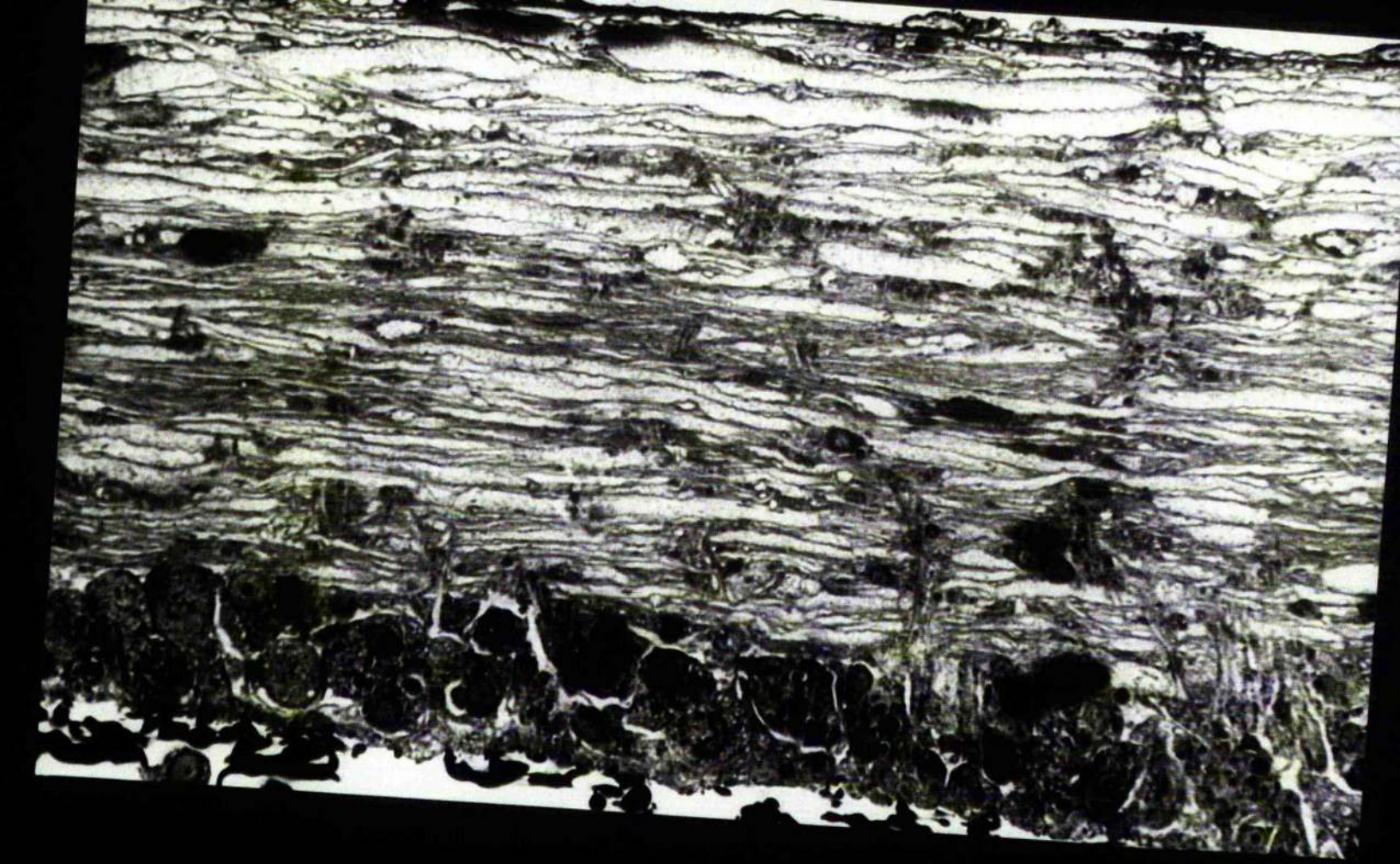
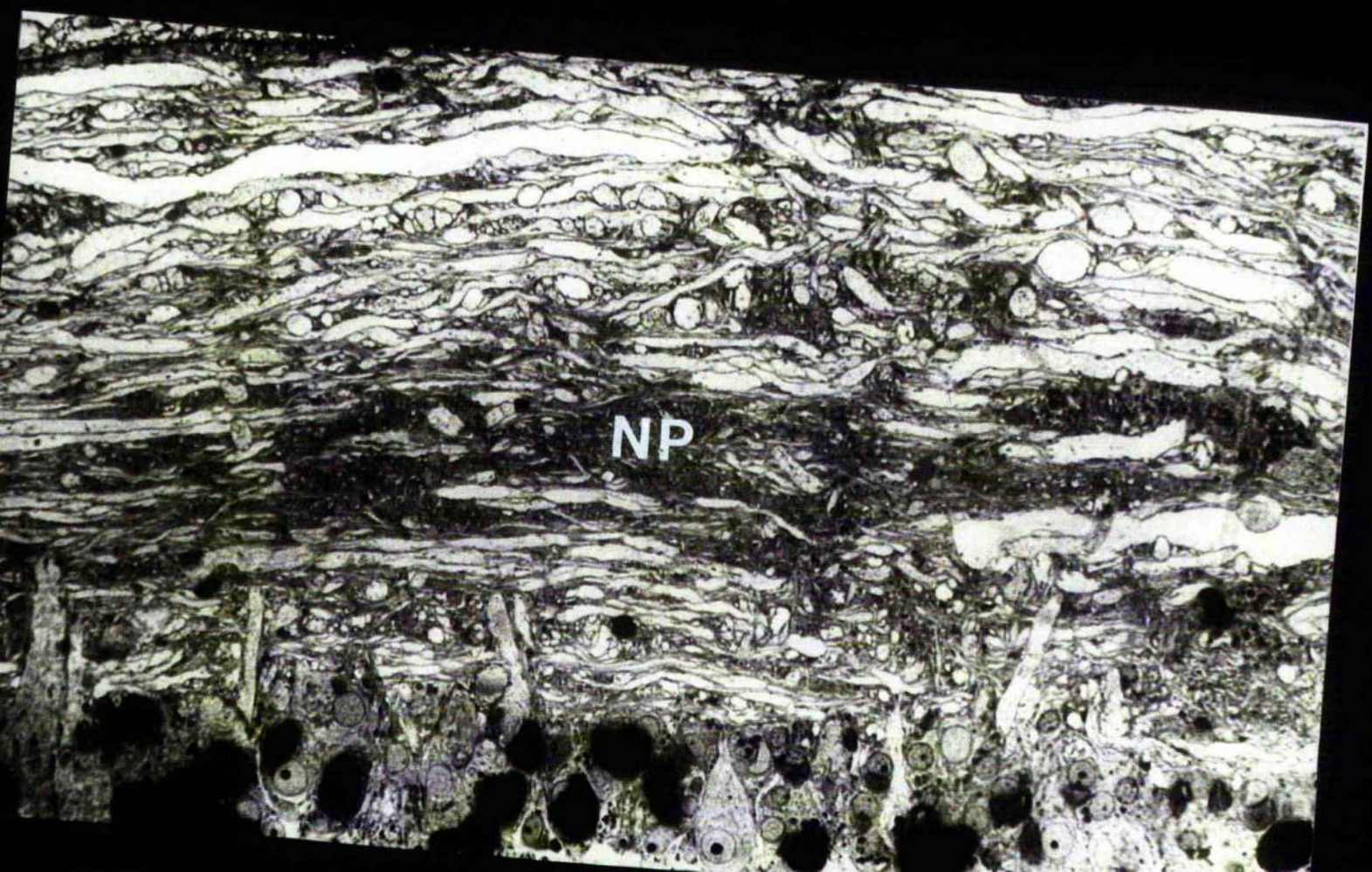

S

Fig. 9

Longitudinal sections of radial nerve cord to show the differences between the ganlionic and interganglionic regions of the nerve cord.
a) The interganglionic regions contain fibres that are aligned in parallel
b) The ganglion regions contain areas of neuropil (NP). There is considerable interweaving of the fibres and some fibres run transversely.

x 400




NP

80 to 100 giant nerve cells. The hyponeural neurones are formed in two groups, one on each side of the midline of the radial nerve cord. One process from the cell body forms the long motor axon and the other breaks up to form a plexus of fine processes (1 μm in diameter) which abuts onto the basal lamina (Figs. 7 and 10). This plexus lies on the opposite side of the basal lamina to the vesicle-filled ectoneural nerve endings. The basal lamina is about 20 nm thick in this region, and this area is presumed to be the site of synaptic contact between the purely motor hyponeural tissue and the ectoneural tissue (Fig. 11).

Circumoral Nerve Ring

The circumoral nerve ring consists of a series of curving connections joining the radial nerves rather than a geometrical ring to which branches are attached. In common with the radial nerves the circumoral nerve ring is composed of ectoneural and hyponeural tissues separated by a basal lamina. The circumoral ring has a circular profile compared to the flattened shape of the radial nerve cords and the hyponeural tissue forms a crescent on the outside of the more bulky ectoneural tissue, rather than being localised into a series of ganglia as in the radial nerves (Fig. 12).

Fig. 10

Diagram of a transverse section through the ganglion region of a segment of a radial nerve cord. The epineural sinus (EP) underlies the ectoneural part of the nerve cord, the oral surface of which is composed of epithelial cells (EC) and ectoneural cell bodies (EN). Axons that run vertically (EN2) bend at right angles around the longitudinal giant axons (LG) and then run transversely. Some longitudinal neurones form endings against the basal lamina (BM). The hyponeural cell bodies are divided into two groups by the radial haemal system (RH) and the radial water vascular canal (RV). Hyponeural cell bodies (HN)

Not to scale

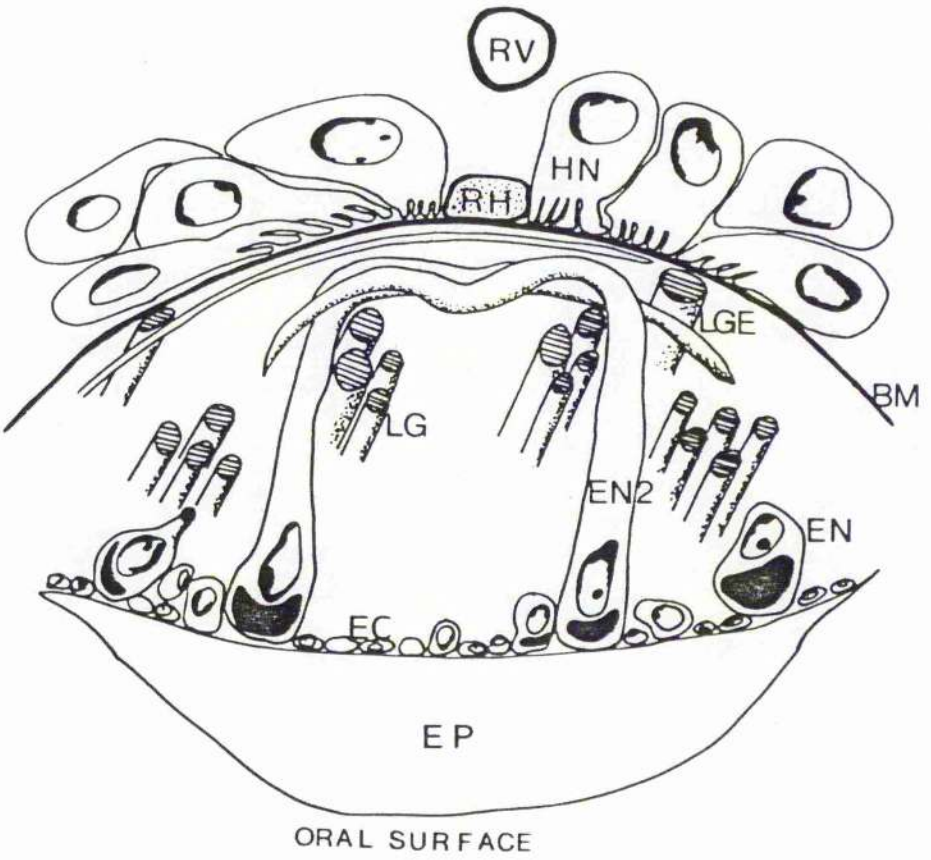

RV
HN
RH
LGE
BM
LG
EN2
EN
EC
EP
ORAL SURFACE

Fig. 11

The hyponeural (HYP) and Ectoneural tissues are separated by a basal lamina (arrowed). Synaptic contact between the two tissues occurs across the basal lamina. Note the vesicle-filled ectoneural nerve endings.

x 7,500

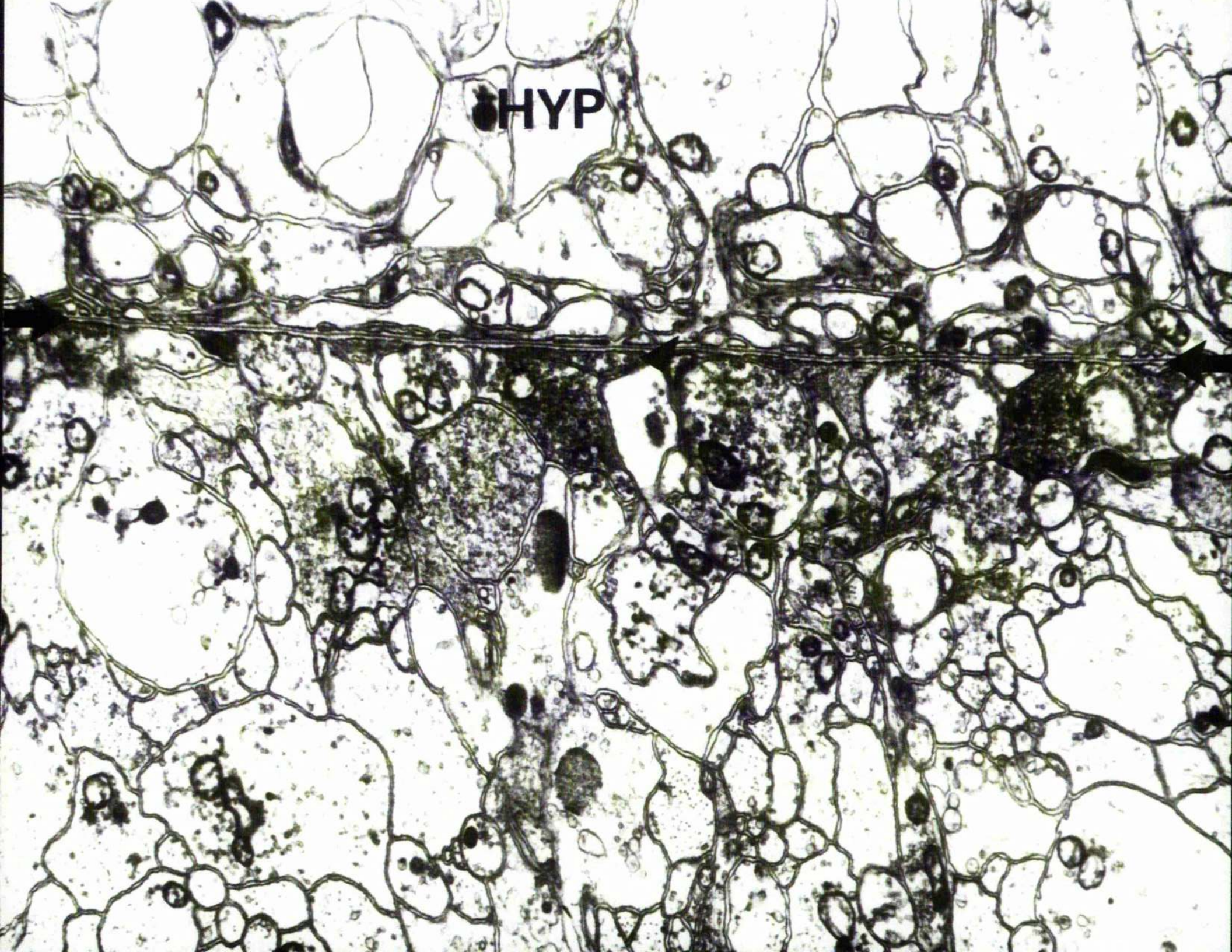
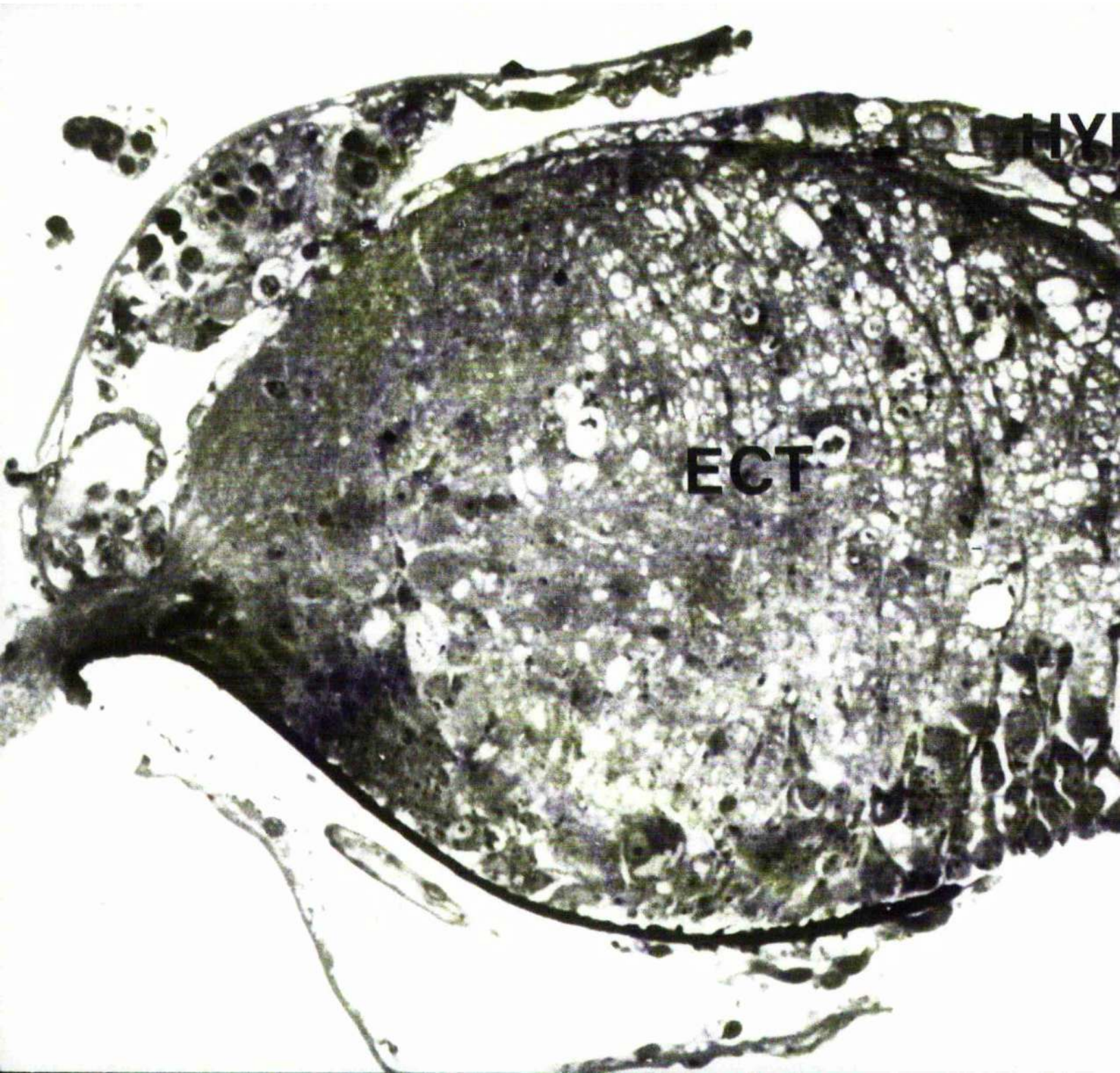

HYP

Fig. 12

Transverse section of a circumoral nerve ring. The hyponeural tissue (HYP) lies in a crescent-shaped profile around the edge of the ectoneural tissue (ECT).

x 250


ECT

The junction regions between the the radial nerve cords and the circumoral nerve ring have been considered as possible sites for the location of controlling centres within the nervous system (Smith, 1966). Horizontal sections of this junction region demonstrate that the number of giant nerve cells is similar to the number found in each regular segment of the radial nerve cord. The orientation of the cell bodies and axons is also strikingly similar to the layout found in the segmental ganglia, with only a slight alteration in pattern to accommodate the divergence of the circumoral nerves. The absence of extensive areas of neuropil suggests that this junction region is unlikely to be the site of major integratory activity. The importance of the structure of this junction region will be discussed in greater detail later in this chapter.

The connections between the radial nerves consist of nerve fibres which are orientated in parallel and which longitudinally follow the curve of the nerve ring. Some twenty to thirty of these fibres are giant and have diameters greater than 10 μm. Areas of neuropil within the circumoral nerve ring are small and are confined to areas where small bundles of fibres leave the nerve ring to innervate the gut and disc.

THE HYPONEURAL MOTOR TRACTS

The main features of the neuromuscular system are illustrated by Fig. 13. The bulk of the hyponeural tissue of the radial nerves is localized over the ganglionic region of the ectoneural part of the nerve cord (see Fig. 2). Each hyponeural ganglion consists of approximately one hundred nerve cell bodies divided into two groups, one on either side of the midline i.e. on either side of the radial haemal canal. The motor axon forms one process from the hyponeural cell, whilst the other process breaks up to form a plexus of fine fibres which are approximately 1 μm in diameter. The motor axon joins similar fibres from other cells to form an axon bundle which penetrates the intervertebral ossicles to innervate the intervertebral muscles (Fig. 14). The hyponeural cells are giant by echinoderm standards with cell body diameters up to 25 μm and axon diameters up to 20 μm. The cytoplasmic structure of the hyponeural cell bodies is similar to the that of the ectoneural neurones with the cytoplasm packed with electron-dense granular material and a marked absence of cellular organelles (see Fig. 6).

The cell bodies comprise the bulk of each hyponeural ganglion, although the ganglion does contain some of the juxtaligamental cells that were first described by Wilkie (1979). However there are no glial cells and the remainder of the ganglion is composed of the network of dendritic fibres from the cell bodies, and two parallel tracts of longitudinally orientated processes from muscle cells (Fig. 15). The network of dendritic fibres lies immediately

Fig. 13

Diagrammatic representation of a longitudinal section through part of two segments of the arm. A group of about 50 nerve cell bodies (cb) lies aboral to the basal lamina (bm) which separates the hyponeural from the ectoneural (ect). A plexus of processes (ph) from the hyponeural cell bodies lies immediately adjacent to the basal lamina. Three main axon trunks (1,2 and 3) leave the hyponeural ganglion and penetrate the vertebral ossicles. A tract of axons connects the ganglia in neighbouring segments (4). The diagram shows only one half of the layout in each segment. A similar group of 50 cell bodies with three axon trunks leaving the ganglion occurs on the other side of the of the water vascular canal, which occupies a position in the mid -line of the arm. A tract of axons (5) joins the two halves of the ganglion. A small branch (6) joins the main nerve trunks (1) on either side of the mid-line. The axon bundles penetrate the juxtaligamental tissue (jl,hatched) and divide to innervate the aboral and oral intervertebral muscles (aim,oim). Nerve 1, the largest nerve, innervates the distal and proximal aboral muscles and part of the oral proximal muscles. Nerve 2 innervates part of the distal oral muscles and nerve 3 innervates part of the proximal oral muscles.

Not to scale

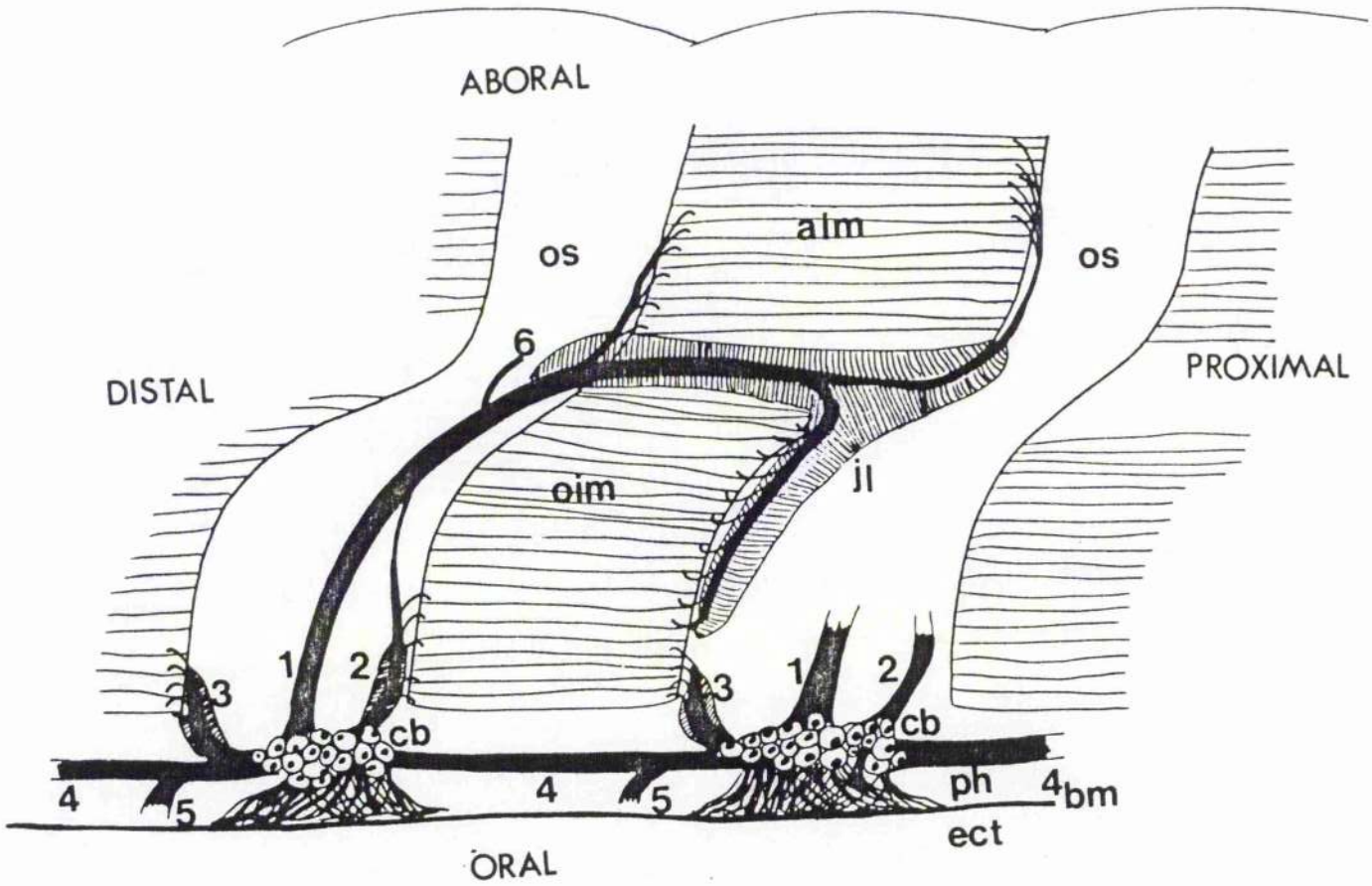

ABORAL
os
aim
os
6
DISTAL
PROXIMAL
oim
jl
1
2
3
cb
1
2
3
cb
4
5
4
5
ph
4
bm
ect
ORAL

Fig. 14

Bundles of motor axons leave the hyponeural ganglia and penetrate the intervertebral ossicles to innervate the intervertebral muscles.
a) Transverse section showing bundles of motor fibres (M) leaving the radial nerve cord (RNC).
b) The bundles of motor fibres (ringed) then penetrate the ossicles (OS) to innervate the muscles.

x 150

Decalcified tissue

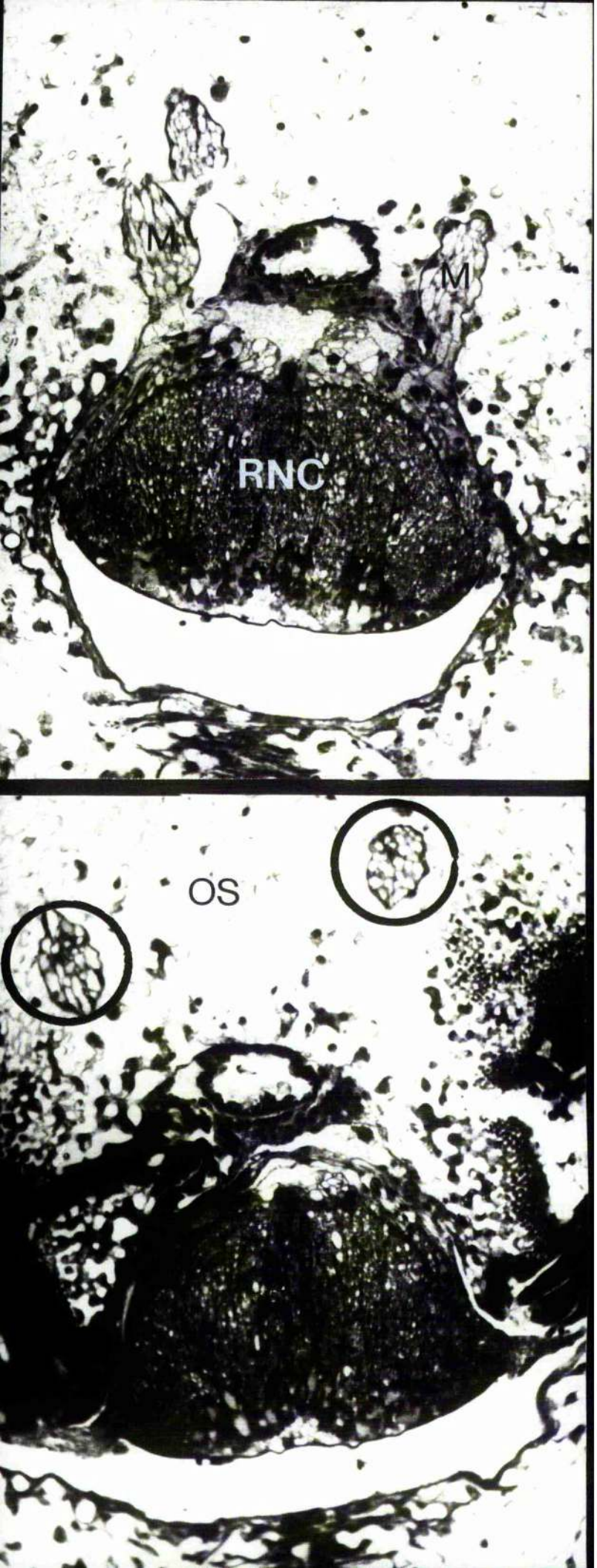
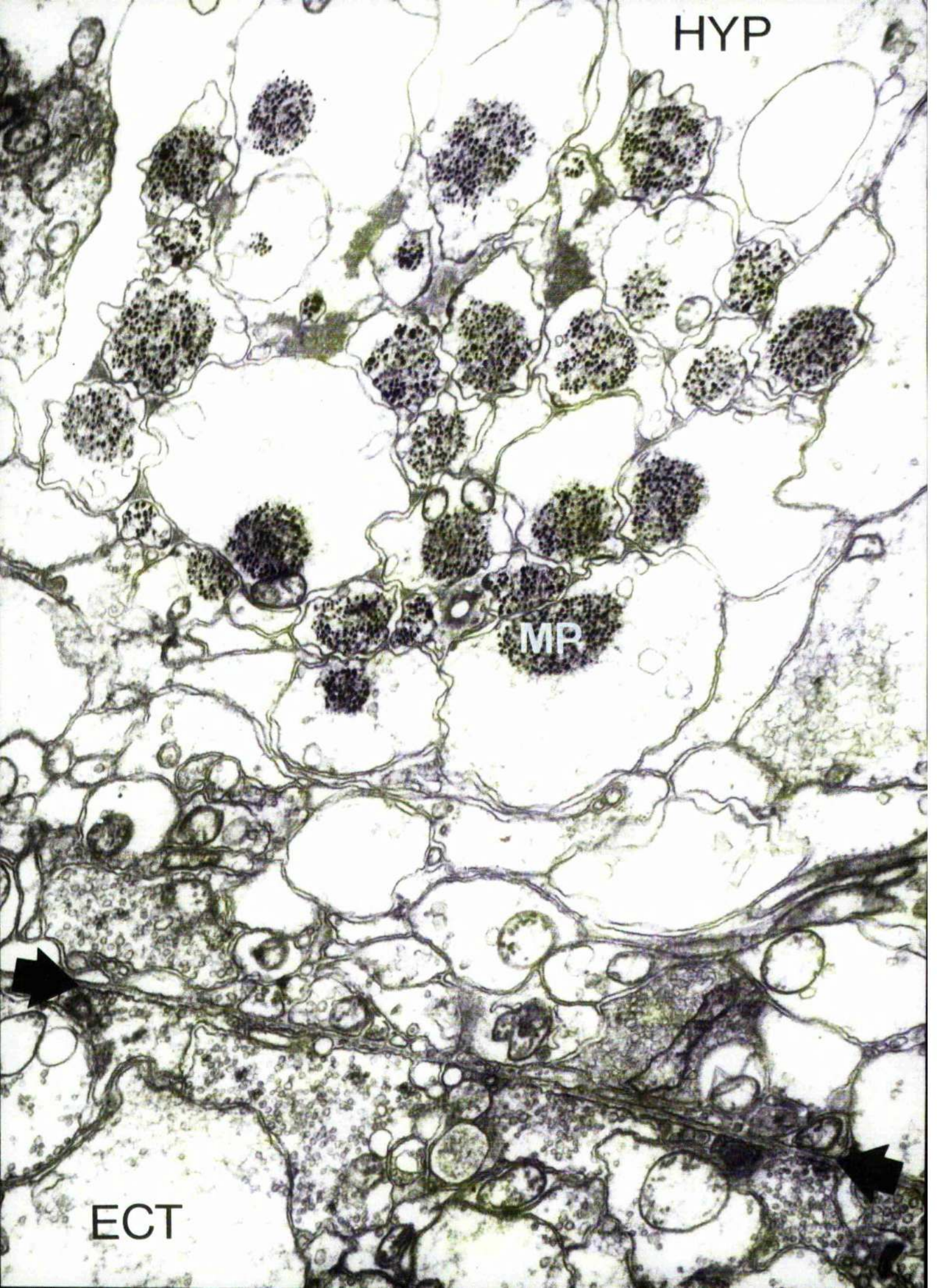

M
M
RNC
OS

Fig. 15

Transverse section through the radial nerve cord showing part of the ectoneural tissue (ECT), the basal lamina (arrows) and one of the pair of bundles of muscle cell processes (MP) that run through the hyponeural tissue (HYP). These processes contain a central core of thick myofilaments.

x 19,000


HYP
MR
ECT

adjacent to the basal lamina that divides the hyponeural tissue from the ectoneural (see Fig. 13). The arrangement of the dendritic network is irregular and the fibres contain small numbers of microtubules and microfilaments. A small proportion contain agranular vesicles 30-50 nm in diameter. There is little evidence of neuropil, although the presence of a few vesicle-filled profiles may indicate limited synaptic activity.

The presence of the two longitudinally orientated tracts of muscle cell processes is enigmatic (Fig. 15). The tracts each consist of twenty to thirty fibres that have diameters of 1 to 2um; they are continuous among the hyponeural tissue on either side of the midline. The fibres appear to originate from the base of the oral intervertebral muscles. The fibres from one segment must overlap with those from the next since the tracts are seen to be continuous. The cell bodies of some of the muscle cell processes are visible adjacent to the oral intervertebral muscles but others must occur within the muscle blocks themselves. These processes have been previously described in detail in other situations in echinoderms (Cobb, 1967b).

Ectoneural-Hyponeural Interaction

Synaptic contact between the ectoneural and hyponeural systems has previously been described in members of the Asteroidea (Cobb,1967b; Cobb and Pentreath,1978). The system in the Ophiuroidea is essentially similar except that some of the

pre-synaptic axons in the ectoneural tissue are derived from giant neurones and are hence large (up to 10um). The presynaptic axons immediately adjacent to the basal lamina (20-30 nm thick) are packed with agranular vesicles (30-50 nm in diameter). The large number of vesicle-filled axon profiles on the ectoneural side of the basement lamina as compared with the hyponeural side suggests that the majority of the synapses operate in an ectoneural to hyponeural direction (Fig. 16).

There are no membrane specializations at these synapses and the pre- and post-synaptic axons are not aligned, but form a two dimensional network on either side of the basal lamina. The unspecialized nature of echinoderm synapses has previously been described by Cobb and Pentreath (1978).

The two bundles of muscle cell processes are, in places, adjacent to the basal lamina and these areas may represent neuromuscular junctions (Fig. 17). Previous work by Cobb (1967b) has demonstrated similar neuromuscular arrangements in other echinoderms. This study shows however that the bulk of the intervertebral muscles are innervated via the hyponeural motor axons (see below) and it therefore seems likely that the muscle cell processes have some other specialized, but as yet, undescribed function.

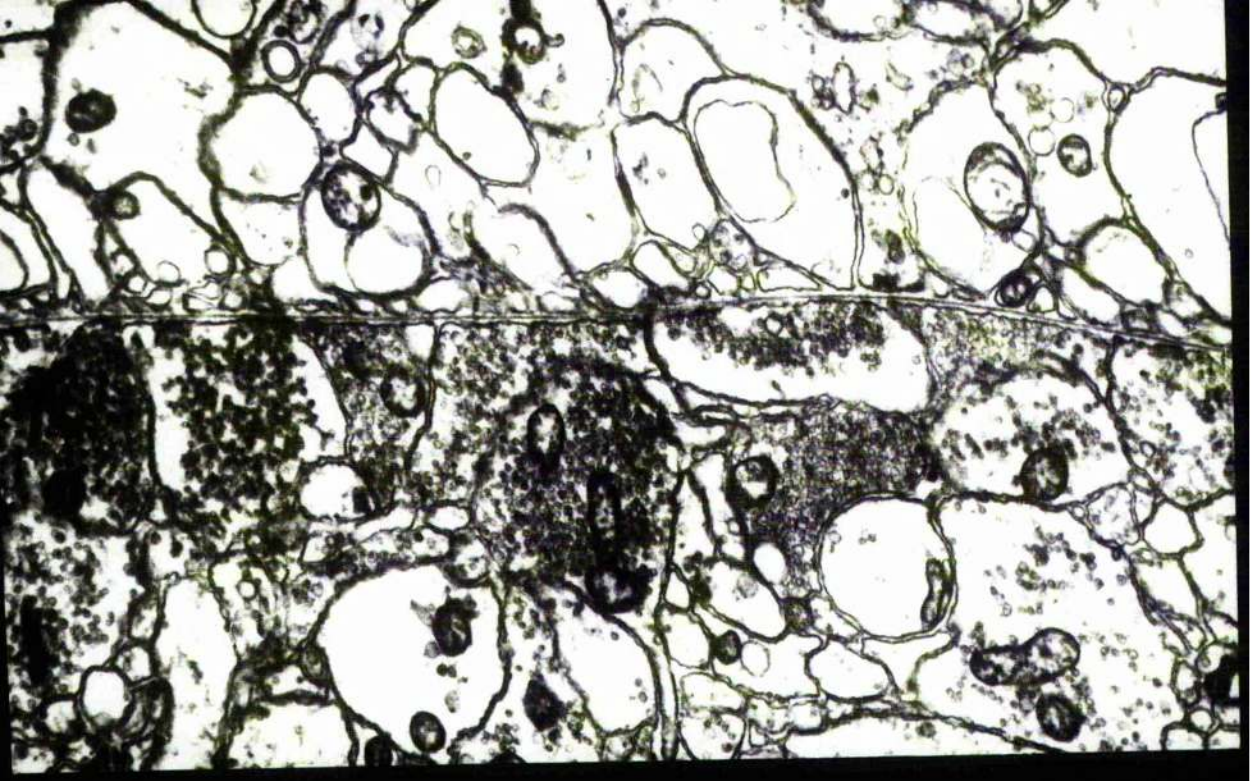
Fig. 16

a) The majority of the vesicle-filled axon profiles are on the ectoneural side of the basal lamina indicating the the majority of the synapses operate in an ectoneural to hyponeural direction.

x 7,500

b) A vesicle-filled ectoneural ending which abuts the basal lamina (arrowed).

x50,000



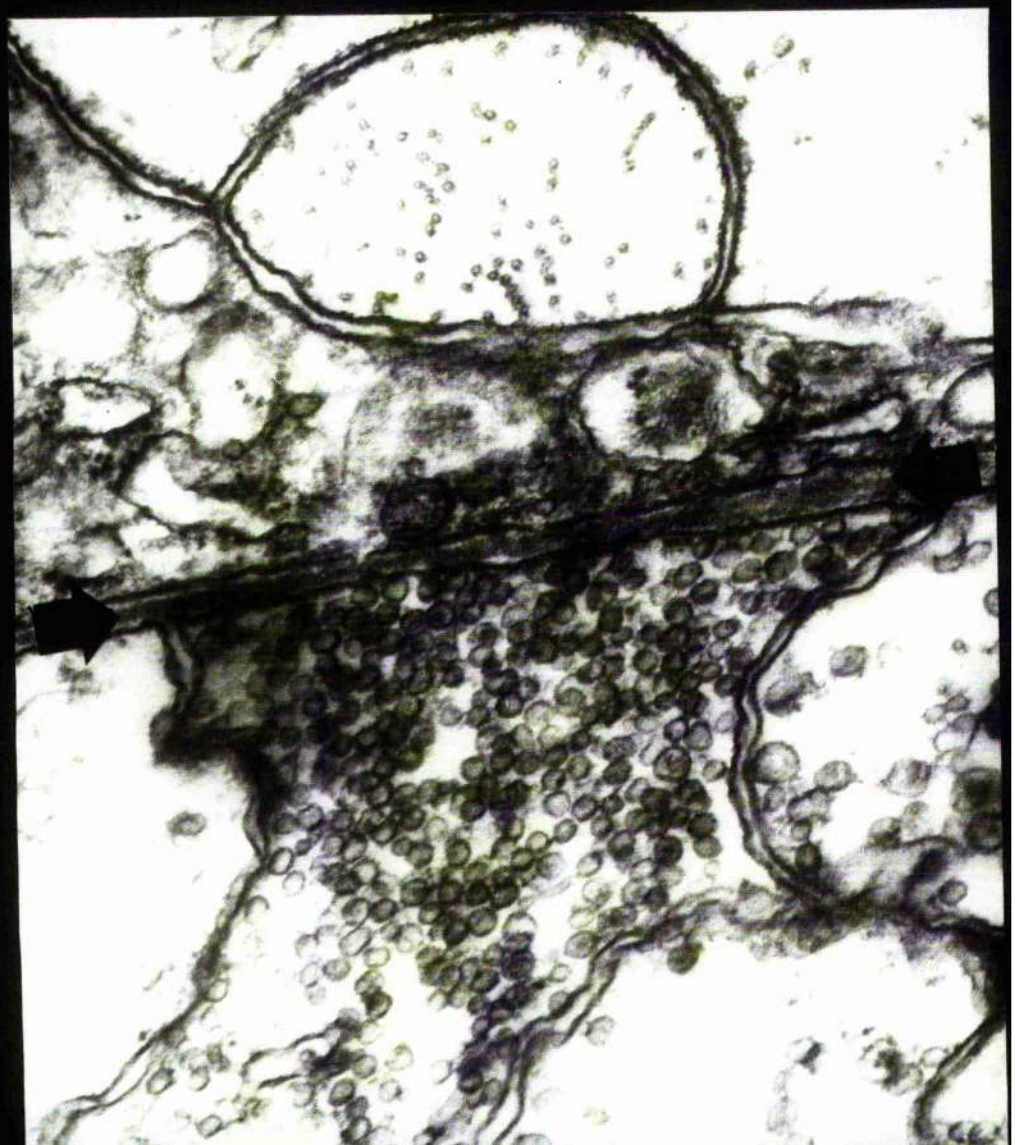
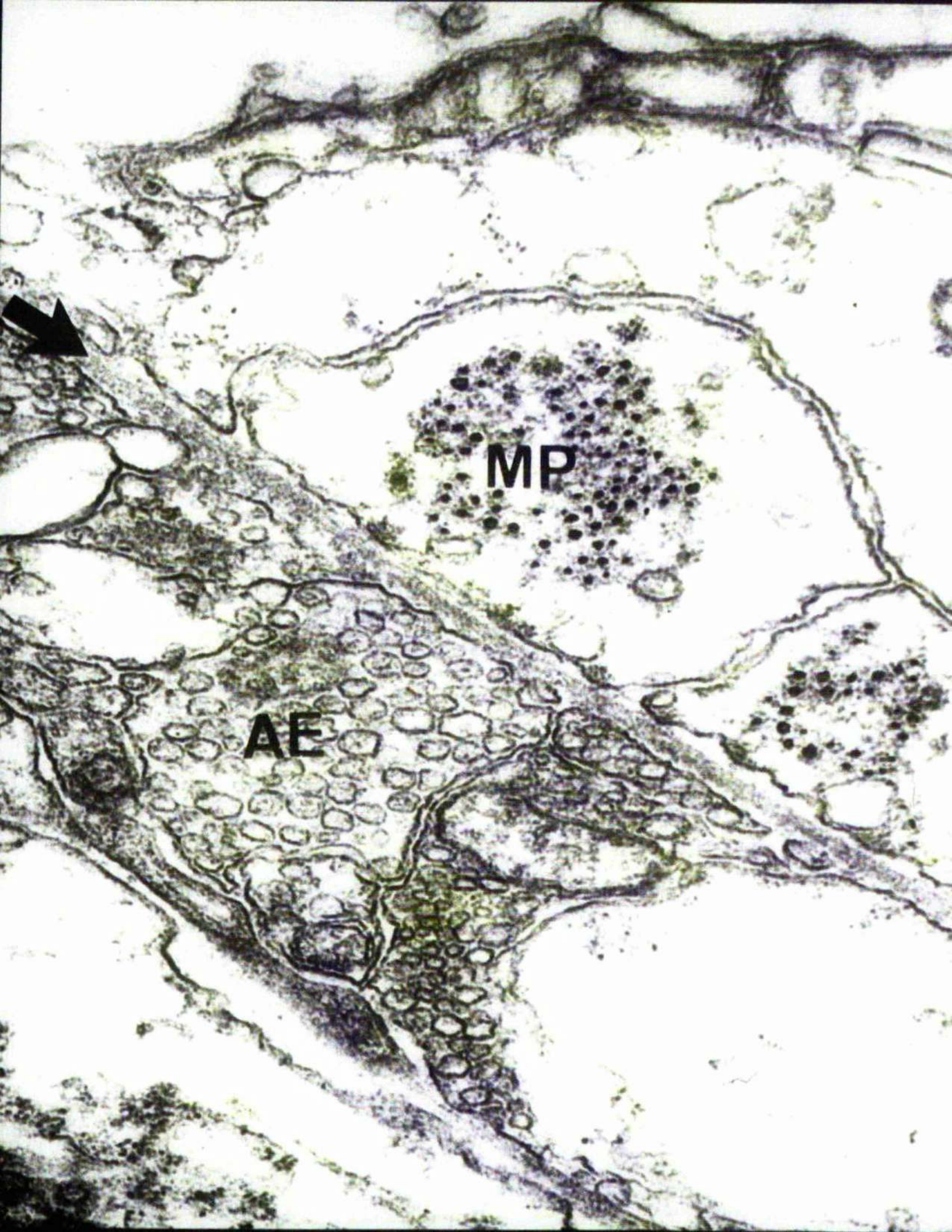


Fig. 17

The muscle cell processes (MP) approach the basal lamina (arrows) and are only separated from the vesicle-filled endings (AE) of the ectoneural system by 20-30 nm. This may well represent a neuromuscular junction (see Cobb,1970).

x 40,000


MP
AE

Pentreath and Cottrell (1971) and Wilkie (1979) state that the basal lamina is not continuous but have failed to publish satisfactory evidence in support of this claim. Investigations using serial sections have shown that in Ophiura texturata there are discrete pairs of fenestrations in the basal lamina at similar sites in each arm segment. At these sites small numbers of axons varying in diameter up to 3 μm pass between the two layers of nervous tissue (Fig. 18). The fibres from the ectoneural do not penetrate the main bulk of the hyponeural tissue but pass aborally, just making contact with the outer edge of the hyponeural tissue. The basal lamina separates the two tissues completely except at these sites, and it seems improbable that these small fenestrations represent sites of major interaction between the two tissues.

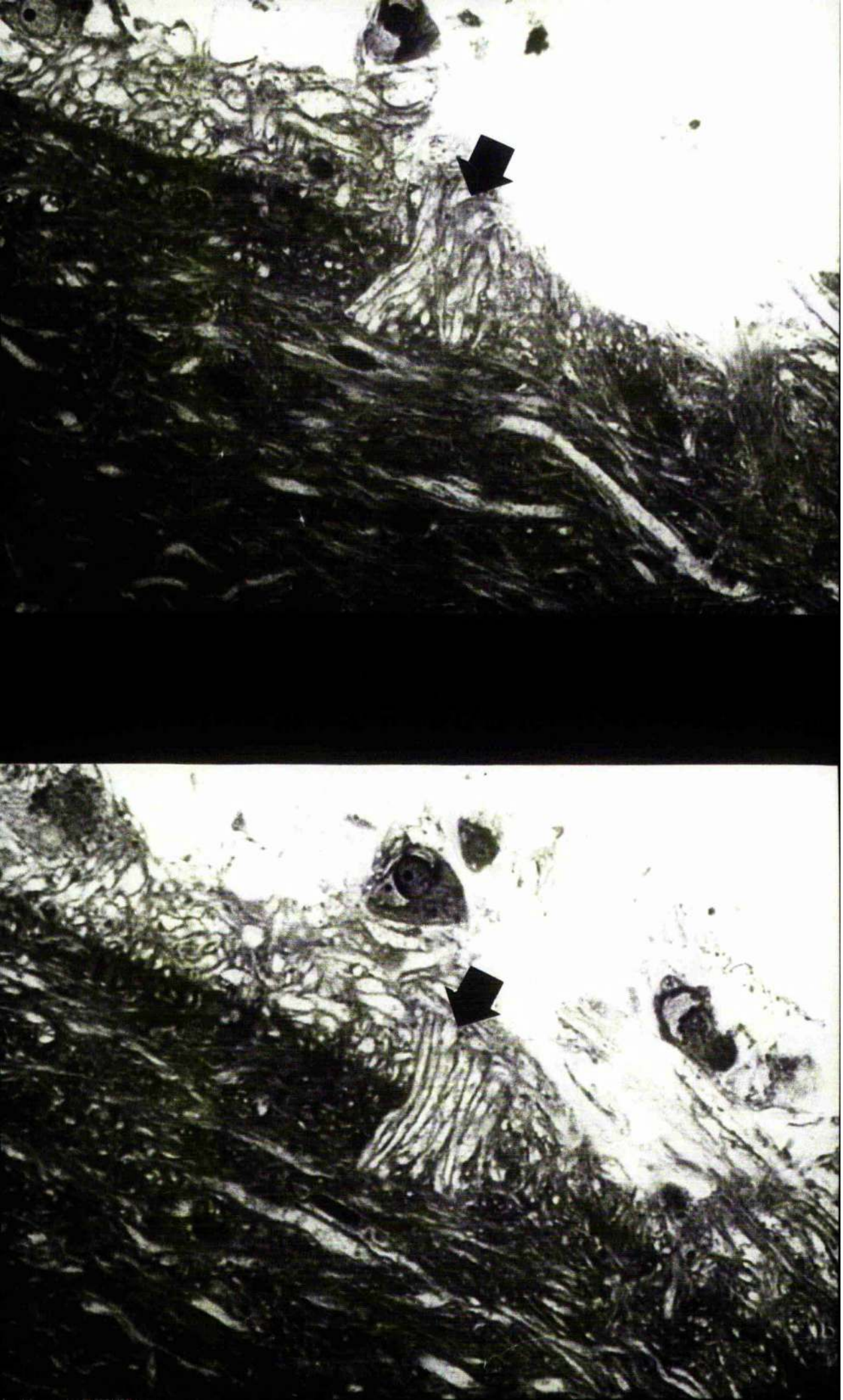
Motor Tracts

Three separate pairs of motor tracts arise from each segmental ganglion. The innervation of the intervertebral muscles by these motor nerves is illustrated by Fig. 13. The largest pair of tracts arise from the middle of each ganglionic swelling of the hyponeural tissue. These tracts each contain about thirty five giant fibres and they innervate most of the proximal face of the oral intervertebral muscle block and a part of the distal face and most of the proximal face of the aboral intervertebral muscle block. Distal to the main trunk is another small pair of axon trunks containing between nine and ten fibres which innervate a

Fig. 18

Light micrographs of longitudinal sections of the radial nerve cord in the region of the main ectoneural side branch. Bundles of axons (arrowed) pass via fenestrations in the basal lamina.

x 450



small part of the distal face of the oral intervertebral muscle. The third pair of axon tracts within each segment arises well proximal to the main trunk and it is more difficult to trace the precise origin of the axons. The majority appear to arise from cell bodies in the hyponeural ganglion of the segment proximal and pass longitudinally down the cord before rising aborally to innervate part of the proximal face of the oral intervertebral muscle.

A tract of hyponeural axons also forms an interganglionic connection. This tract is immediately adjacent to the bundle of axons that forms the third motor tract and there is an intermingling of the fibres between these two axon bundles. It is thus difficult to be certain that none of the interganglionic fibres pass aborally to innervate the muscles. It is clear however, that the majority of the axons in the third tract are not interganglionic fibres. The oral intervertebral muscle is innervated in part by axons from the third motor tract of the ganglion in the arm segment proximal to the muscle block. The majority of the innervation is via the largest motor tract from the ganglion which lies in the same segment as the muscles that are being innervated.

There are tracts of hyponeural axons that cross the midline at a particular point but it is not certain if these arise as branches from motor neurones (i.e. distal to the cell body) or are part of the synaptic mat of fibres (i.e. proximal to the cell body) or are a separate class of interneurones. The latter seems unlikely as

the number of cell bodies in a single ganglion is in good agreement with the number of motor axons observed.

Juxtaligamental Tissue

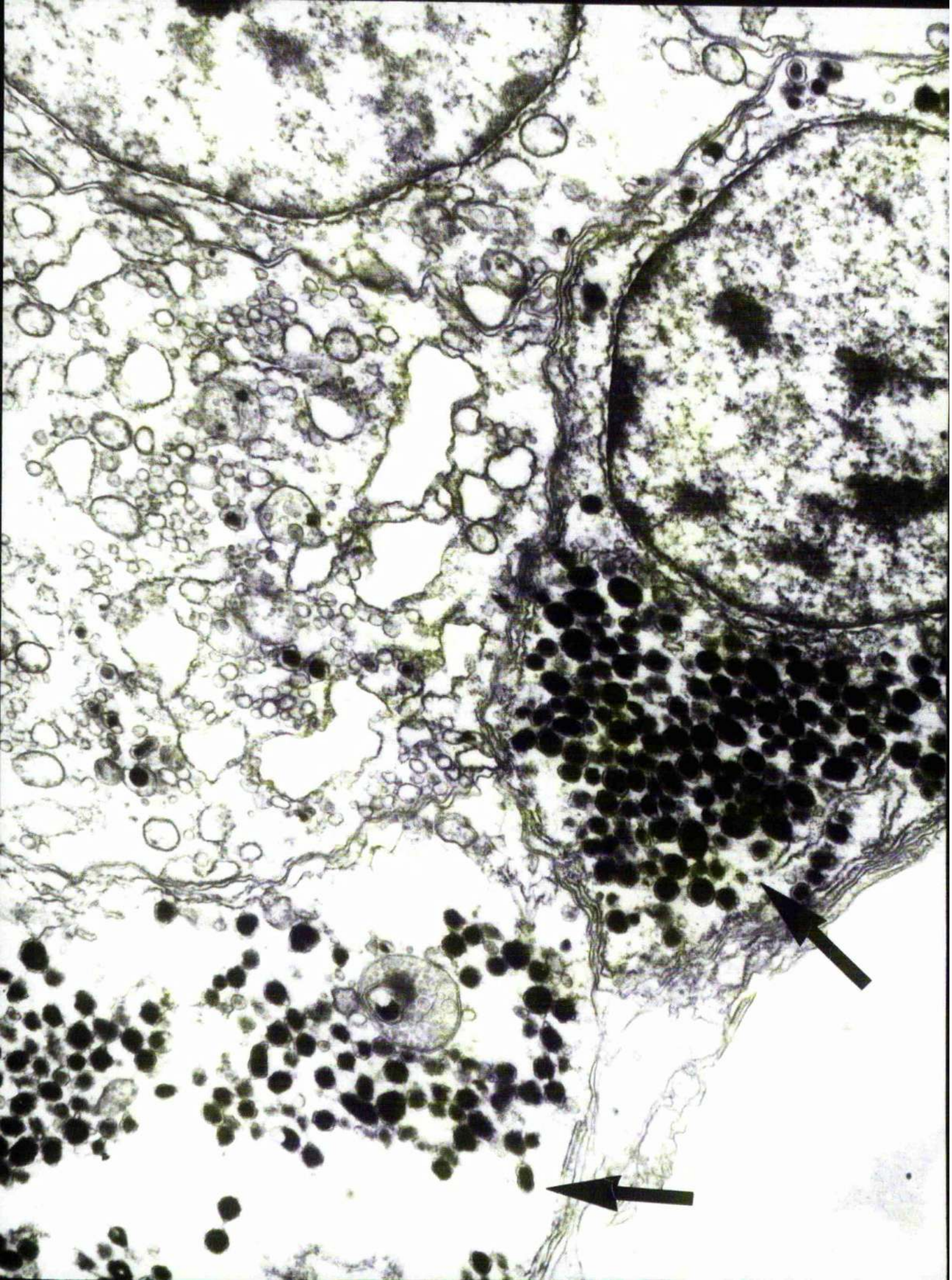
Wilkie (1979) has described the distribution of this tissue and has proposed that it is associated with changes in the structural properties of collagen (Fig. 19). Wilkie described two types of cells, monopolar acidophilic and non-acidophilic. The present work confirms the anatomical details described by Wilkie. The hyponeural giant motor fibres are associated with the juxtaligamental cells close to the regions where they divide to form the pre-terminal fibres. There are two possible types of contact between the giant fibres and these cells. The first contact is formed by axon processes which are filled with agranular vesicles and which are adjacent to processes from the juxtaligamental cells. In some regions the giant axons make very close contact with the soma of the juxtaligamental cells. The quality of fixation when using decalcified material precludes the the identification of gap (electrotonic) junctions with certainty.

The processes from the juxtaligamental cells contain large granular vesicles and these are always associated with regions of collagen, as described by Wilkie, and never invade the muscle region.

Fig. 19

Juxtaligamental cells (arrowed) containing typical large granular vesicles. It is thought that secretions from the juxtaligamental cells influence the plasticity of the collagenous ligaments of the arms, and thus have a crucial role in arm autotomy. Hyponeural motor axons are closely associated with juxtaligamental cells in the region of the innervation of the intervertebral muscles.

x 25,000



Motor fibres

Sections cut well distal to the cell bodies, where the axon bundles pass through the arm ossicles, show that the axons contain small numbers of microfilaments, microtubules and mitochondria, but no other inclusions. There are no small diameter neurones in the motor tracts; all the axons are greater than 3μm in diameter and in large animals may reach a diameter of 20 μm (see Fig. 14). The axon bundles are partially enveloped by processes from interstitial cells. The whole axon bundle is surrounded by an acellular connective sheath about 150 nm thick. The giant fibres of these motor nerves eventually run close to the intervertebral muscles, divide into a number of smaller processes and then innervate a large number of muscle cells in a multiterminate fashion.

Neuromuscular Junctions

The cells of the intervertebral muscles run from a collagenous attachment at one vertebral ossicle to a similar attachment at a neighbouring ossicle. Each cell is elliptical in cross section and about 5μm across at the shortest diameter. The myofilaments are typical of those found in echinoderm smooth muscle (Cobb and Laverack,1966) with a small region of clear cytoplasm surrounding the core of filaments, and containing numerous mitochondria and some smooth endoplasmic reticulum. A small elliptical nucleus lies in an expansion of the cytoplasm usually toward the attachment

region at one end or the other. There appear to be no specialized connections such as gap junctions between the muscle fibres which implies that they are all directly innervated. The giant axons penetrate the muscle blocks and divide into a number of pre-terminal axons of smaller diameter or alternatively they are often seen to divide just before they penetrate the muscle block. These pre-terminal axons are between 2 and 4μm in diameter and contain extensive microtubules and microfilaments. Bundles of between three and seven terminal axons run at right-angles to the direction of the muscle fibres. These bundles are found about 20 μm to 30 μm from the muscle attachment to the vertebral ossicle (Fig. 20). The terminal regions of the motor axons are characterised by substantial concentrations of agranular vesicles which are between 35 and 50 nm in diameter. Some of the fibres show varicosities typical of vertebrate autonomic endings. All the terminal axons pass many muscle fibres, usually penetrating between two adjoining muscle fibres and apparently making synaptic contact on both sides (Fig. 21). The synapses at the neuromuscular junction are non-specialized as is the invariable rule in echinoderms (see Cobb and Pentreath,1978). Some muscle cells have small processes that partially enclose the terminal axons as has been previously described (Cobb and Laverack,1966). All regions of both the oral and aboral intervertebral muscles are innervated in this fashion. They appear to be innervated at one end only and this depends on the particular giant fibre tract involved.

Fig. 20

The pre-terminal regions of the motor axons are formed by the division of the "giant" motor axon into a large number of smaller axons. These pre-terminal axons are characterised by the presence of many microtubules and a few small agranular vesicles. They penetrate the muscle blocks and invariably run at right angles across the muscle fibres (M). The pre-terminal axons (PT) are larger , contain more microtubules and fewer vesicles than the terminal regions of the axons. There are no specialisations of the pre- or post-synaptic membrane. Decalcified tissue.

x30,000

Fig. 21

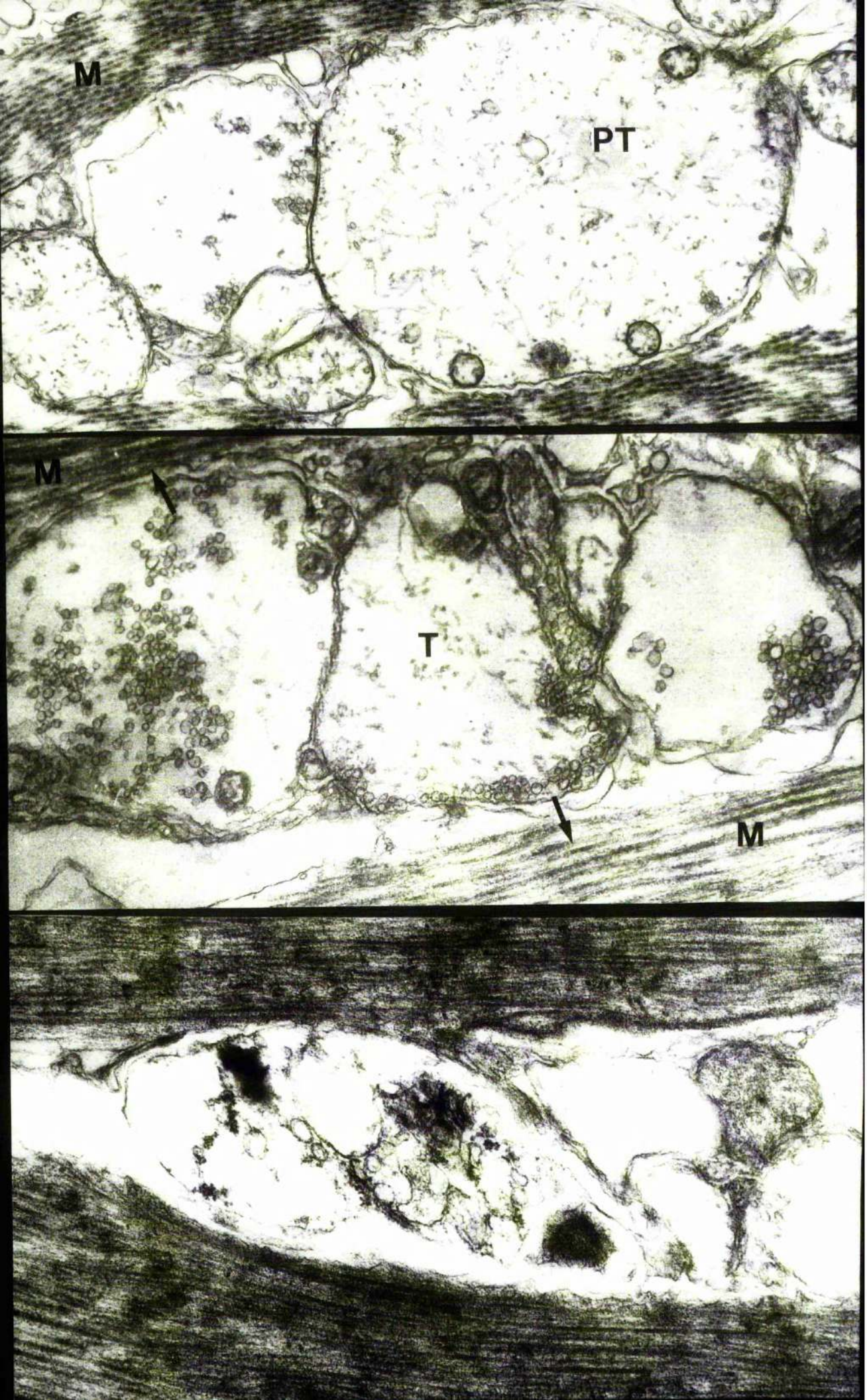
Transverse section through the terminal region (T) of axons which contain numerous small agranular vesicles. Each axon makes synaptic contact (arrows) with a large number of muscle cells (M). Decalcified tissue.

x 30,000

Fig. 22

Transverse section of a degenerating hyponeural motor axon terminal within the intervertebral muscle block. The radial nerve cord and motor axon somata had been removed six days prior to fixation. All motor axons in this material show unequivocal evidence of degeneration, the main axons as well as the terminal region. Decalcified tissue.

x 30,000


M
PT
M
T
M

Degeneration studies were carried out to confirm that the giant fibres did divide into a large number of pre-terminal axons rather than synapse onto a second order of motor neurones. Animals were examined five to nine days after destruction of the hyponeural cell bodies, by ablation of a section of the radial nerve cord. The cytoplasm of the giant axons had become completely electron lucent and the membranes were irregular and broken; there were small aggregations of flocculent material and occasional lysosomes. The pre-terminal fibres appeared similarly degenerated but contained substantially more lysosomes and in some cases the degeneration was total. The terminal regions of the axons were either absent, packed with lysosomes or consisted merely of fragmented membranes (Fig. 22).

THE CIRCUMORAL NERVE RING AND ITS CONNECTIONS

The details of the layout of the axon pathways described below are summarised in Fig. 23. The circumoral nerve ring, like the radial nerve cords, is composed of ectoneural and hyponeural tissues separated by a basal lamina (see Hyman,1955). The nerve ring is circular in cross section and for the purposes of this description is divided into oral, outer, aboral and inner quadrants (see Fig. 24). The hyponeural tissue appears as a crescent-shaped profile on the outside of the ectoneural tissue. The nerve ring is bounded and enclosed by the connective tissue of the epineural and hyponeural ring sinuses.

Ectoneural Tissue

Circumoral Ring

Examination of transverse sections of the circumoral nerve ring with the light microscope revealed that the cell bodies of the ectoneural tissue are largely confined to the lower part of the oral quadrant and are covered by a layer of small epithelial cells which are joined by desmosomes. Some of the cell bodies contain modified cilia (Fig. 25), as do some of the hyponeural cell bodies. Gardiner and Rieger (1980) have described modified cilia in echinoderm muscle cells and have suggested that they may have a sensory function. The vast majority of the axons are less than 1.5 um in diameter, but approximately 40 are an order of magnitude larger. Three or four of these axons are 20 um in diameter in

Fig. 23

Two dimensional diagram (oral view) to illustrate the main nerves of the circumoral nerve ring (COR) and the proximal part of the radial nerve cord (RAD). There are two pairs of modified buccal tube feet (BP) near the mouth. The radial muscles (RM), the external inter-radial muscles (EIM) and the internal inter-radial muscles (IIM) are all innervated by branches from the hyponeural tissue (9,10 & 11) The jaw muscles (JM) are however innervated by an ectoneural nerve branch (1). The teeth of the jaws are innervated by a branch (7) from the ring ganglion of the first buccal tubefoot. Other ectoneural nerve branches innervate the tube feet (BP,P)(branch 2), the peripheral regions of the arm (branches 3 & 4), the peripheral regions of the disc and gonads (branch 6) and the gut (a pair of nerve branches 8). A branch from the hyponeural tissue (5) penetrates the basal lamina via a fenestration, to innervate the oral ligaments.

Not to scale

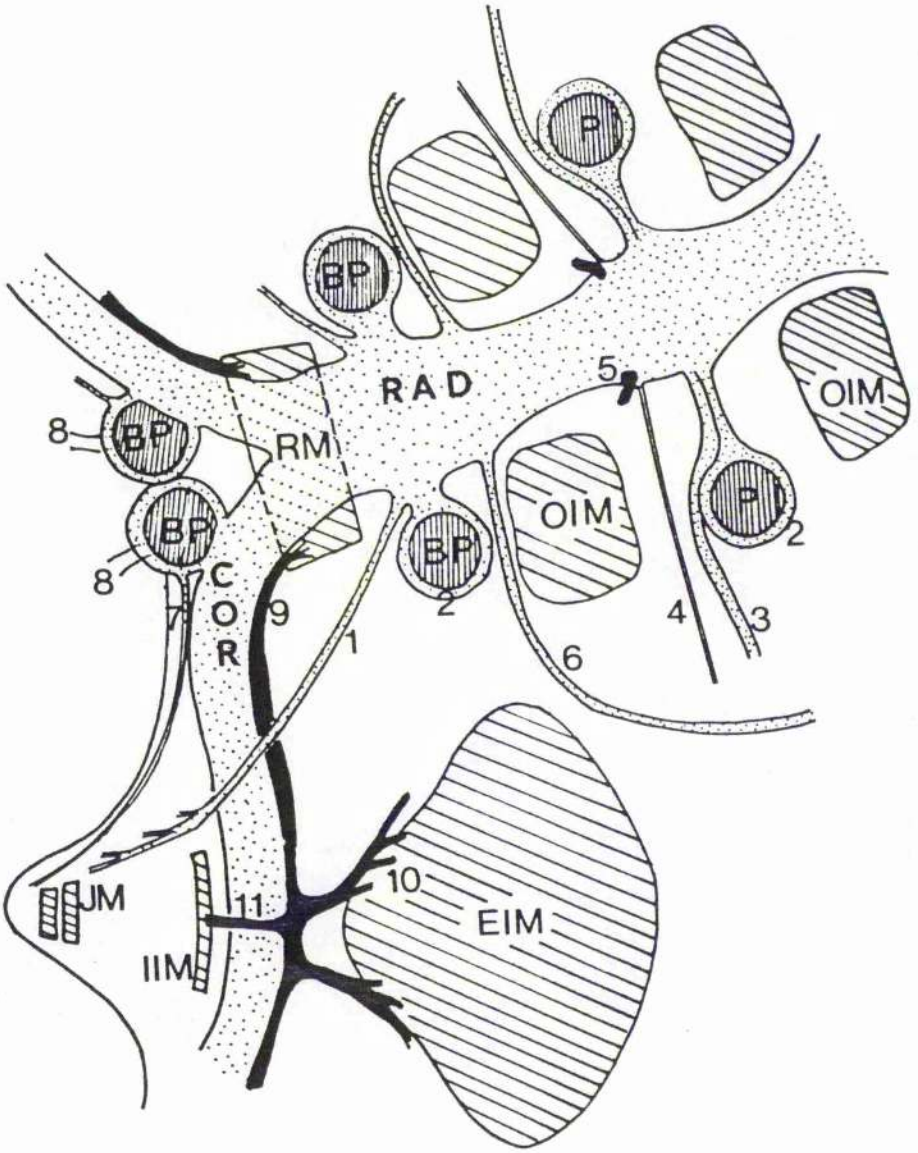

P
BP
RAD
5
OIM
8
BP
RM
BP
8
C
O
R
9
1
BP
2
OIM
P
2
4
3
6
JM
11
10
EIM
IIM

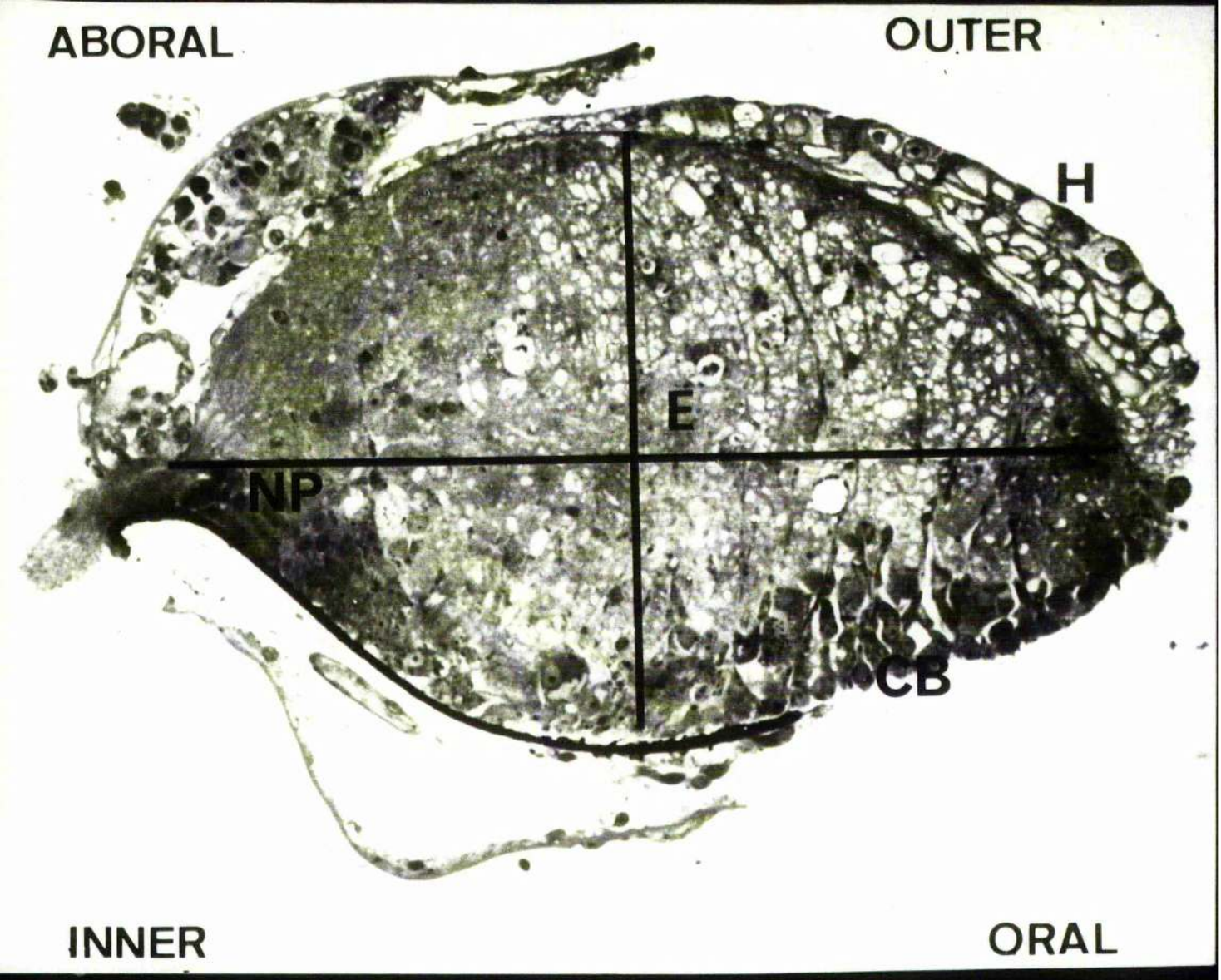
Fig. 24

a) Light micrograph of transverse section through the circumoral nerve ring. The cell bodies (CB) of the ectoneural tissue(E) lie mainly in the outer and oral quadrants. The hyponeural nerves (H) and their cell bodies lie on the outside of the ectoneural tissue. Some of the small diameter fibres form discrete areas of neuropil (NP).

x 250

b) Some of the giant ectoneural fibres show degeneration (arrows). The flocculation of material within the cytoplasm readily distinguishes degenerating axons from non-degenerating axons.

x450


ABORAL
OUTER
H
E
NP
CB
INNER
ORAL

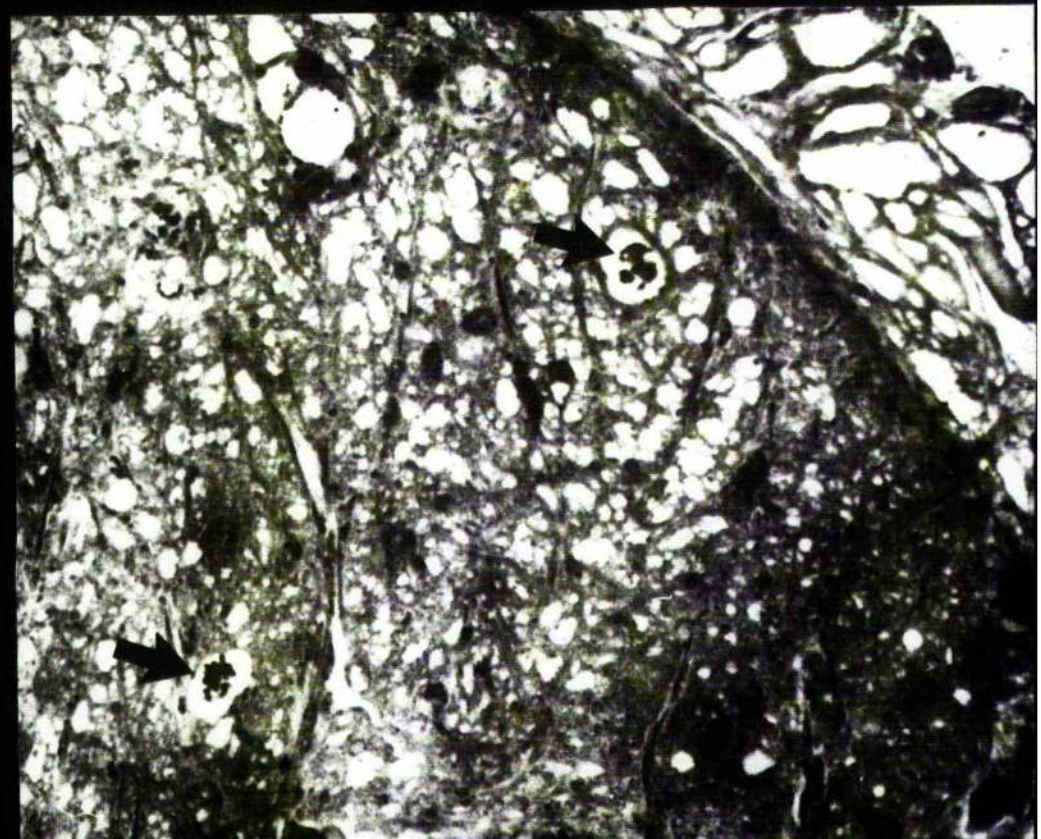


Fig. 25

Modified cilia are present in the cell bodies of many of the ectoneural and hyponeural neurones in both the radial nerve cords and circumoral nerve ring. The functional significance of the ciliation of these neurones is unclear.

x 20,000

large specimens (above 2 cm disc size), the remaining large axons average 10 um in diameter. All the giant axons and most of the smaller ones run circumferentially in the nerve ring (Fig. 26). There are no giant fibres that run vertically, diagonally or transversely as they do in the radial nerve cord ganglia. The cytoplasmic structure of the cell bodies is similar to that already described for the radial nerve cords. In material that was not block-stained the cell body cytoplasm was filled with electron dense granular material as were a small proportion of the axon profiles (Fig. 27). This granular material was not present in block stained tissue; the disappearance of the granular material with block staining was reproduceable in both the circumoral nerve ring and radial nerve cords. This granular material may be glycogen although its origin is unclear; there is an apparent lack of endoplasmic reticulum and other organelles within the cell.

The ectoneural tissue contains small areas of neuropil throughout the inter- radial region of the nerve ring in the inner quadrant (Fig. 24), but these areas of neuropil do not involve the giant fibres nor are they as extensive as the neuropilar regions of the radial nerve cords. The areas of neuropil are characterised by axons packed with vesicles. The axons show a random orientation and many are clearly varicose. Presumed synapses (Fig. 28) do not show specializations associated with the membrane or the cytoplasm either pre- or post-synaptically.

Fig. 26

A longitudinal section through part of the circumoral nerve ring showing the parallel arrangement of the fibres.
E - ectoneural tissue
H - hyponeural tissue

x 450


H
E

Fig. 27

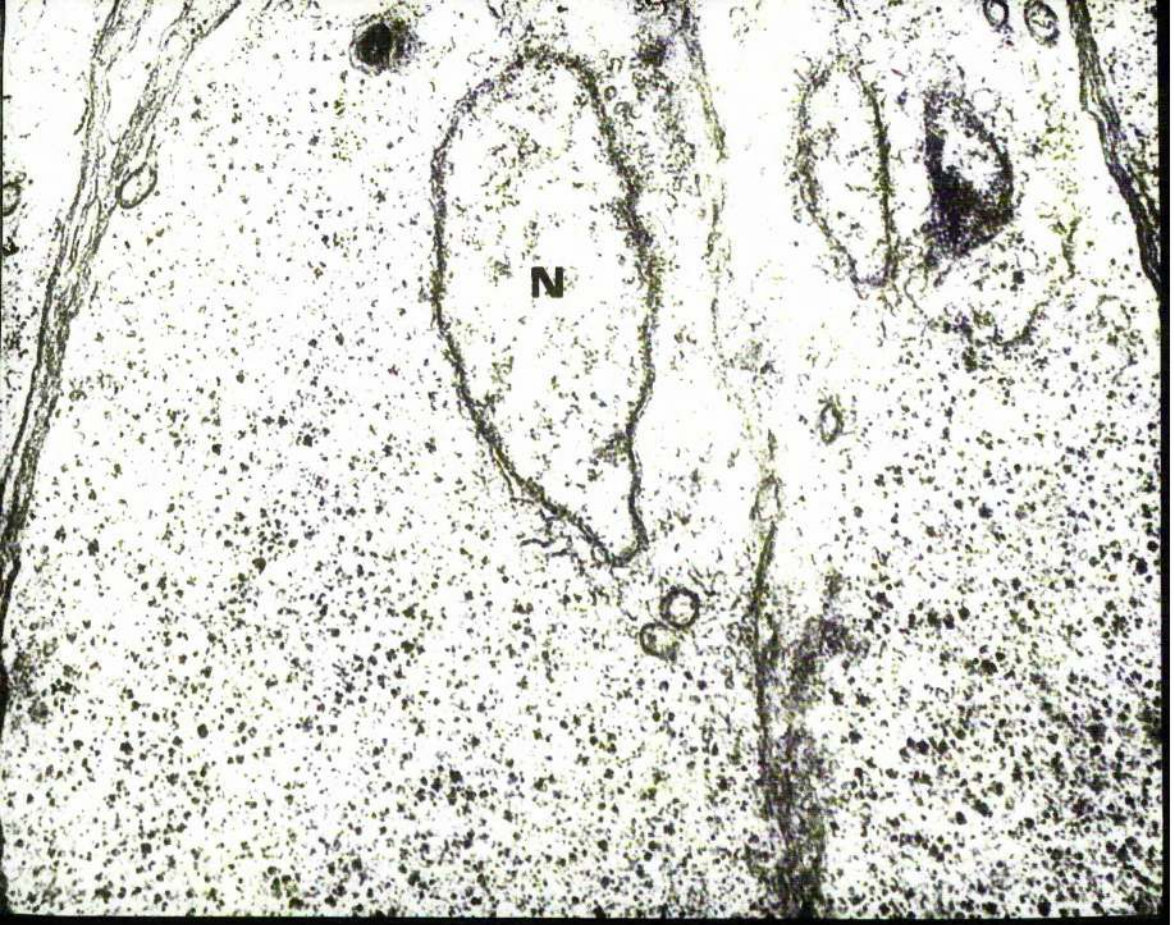
Cell bodies (N = nucleus) in the ectoneural tissue showing a considerable quantity of electron dense granulation within the cytoplasm and little endoplasmic reticulum. This granulation is not seen in tissue that has been treated with aqueous uranyl acetate block stain after fixation.

x 7,000

Fig. 28

The circumoral ring contains regions of neuropil with non-specialised synapses. Profiles, such as that illustrated, showing the vesicles in regular alignment are rare and even in cases where this does happen there are no pre- and post-synaptic membrane specialisations.

x 50,000


N

Nerve Branches from the Circumoral Ring

There are a number of small axon bundles that pass into the ossicles of the disc and jaws (Fig. 29), and some of these contain only small fibres less than 1 μm in diameter, whilst others contain fibres up to 2 μm in diameter. The ectoneural nervous system is considered to be both sensory and motor but there is, as yet, no anatomical or other evidence that allows a distinction to be made between these functions in any part of the nervous system. This clearly poses considerable problems in the interpretation of the functions of the nerve pathways. Five main pairs of ectoneural nerve bundles innervate the gut. The regions of neuropil in the ectoneural tissue of the nerve ring appear to be mainly associated with these minor nerve tracts.

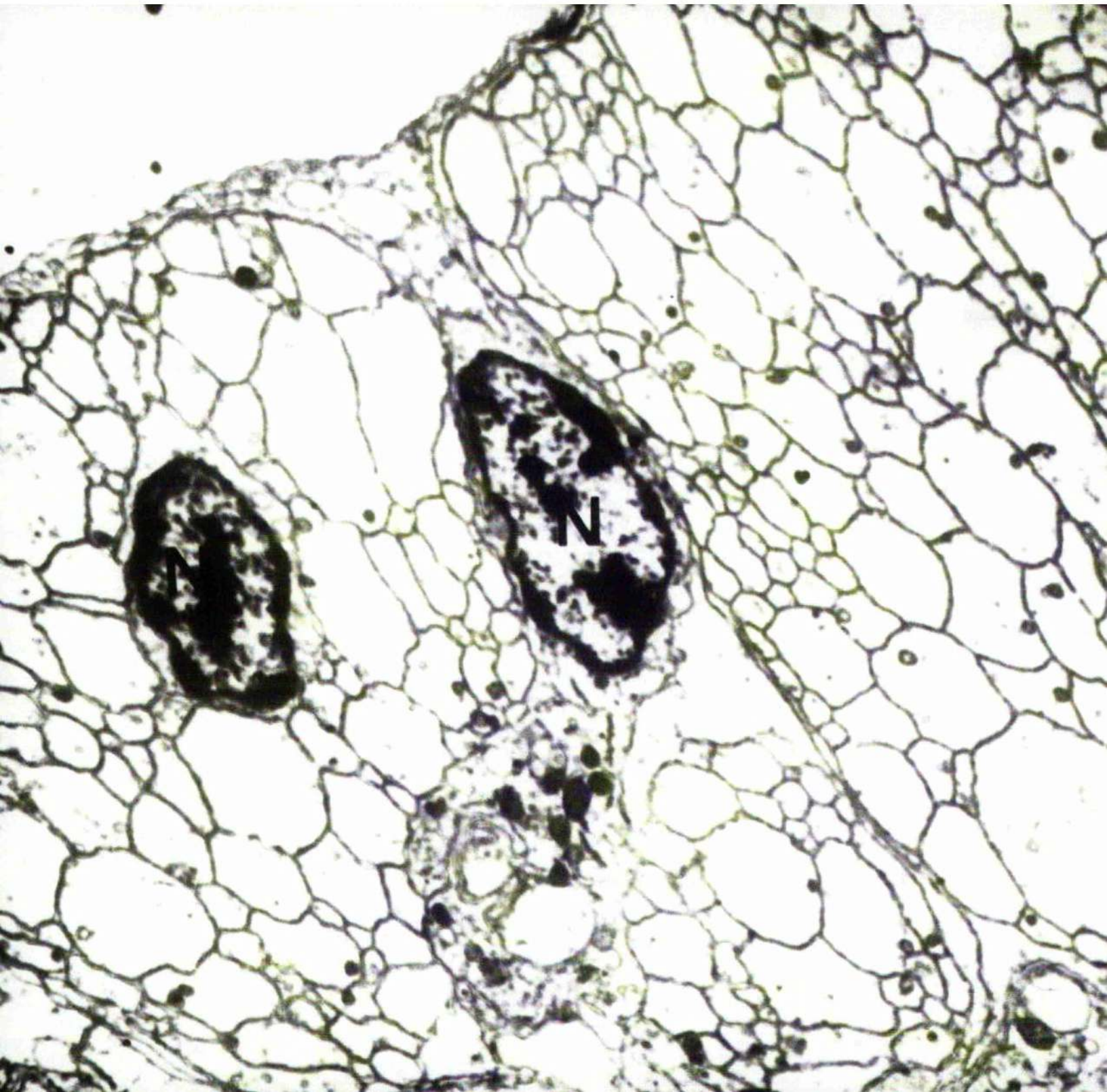
Innervation of the Buccal Tube Feet

The first two pairs of tube feet in each radius, known as the buccal tube feet, have a different innervation from the rest of the tube feet of the arms. This innervation is illustrated diagrammatically in Figure 24. Each tube foot is innervated by an ectoneural nerve branch which forms a ring of nerve tissue that encircles the base of the tube foot. This has been previously described as a ring ganglion (Hyman,1955). In the case of the first pair of buccal tube feet the ring ganglion surrounding the base of the tube foot is very close to the circumoral nerve ring. There are a number of distinct branches from this ring ganglion of

Fig. 29

Transverse section of part of a small bundle of ectoneural axons which passes from the ring nerve of one of the first pair of buccal tube feet to the muscles of the jaw. The nuclei of two of the support cells can be seen (N). The relatively large size of the axons suggests that this particular bundle may be purely a motor nerve branch. Decalcified tissue.

x 7,000


N
N

the buccal tube feet. These branches innervate the maxilla and the teeth of the jaws.

The second pair of tube feet are each innervated by the usual pair of ectoneural nerve branches, one of which forms the ring ganglion at the base of the tube foot and the other continues past the tube foot to innervate the wall of the arm. This latter nerve branch innervates most of the ambulacral and adambulacral plates which form the disc. There is a third segmental ectoneural nerve branch, which is usually a minor lateral nerve, but is in this case enlarged and it innervates the genital structures and many of the inter-radial disc structures.

Hyponeural Tissue

Circumoral Ring Structure

One of the main differences between the circumoral nerve ring and the radial nerve cords is that the hyponeural tissue in the oral nerve ring is spread out in a crescent-shaped profile, rather than being aggregated into the series of ganglionic swellings, which are found in the radial nerve cords. The hyponeural tissue lies outside the ectoneural tissue with the cell bodies, which are bipolar, in a circumferential strip at the top of the oral quadrant. The strip of cell bodies runs from the junction with the radial nerve cord in both directions, and these strips do not quite meet inter-radially. One process from the cell body forms the large motor axon which passes either radially or inter-radially in a circumferential tract. The other process breaks up to form a

plexus of small endings which abut onto the basal lamina. There are ectoneural axons on the opposite side of the basal lamina which are packed with synaptic vesicles, and it is clear that this represents a region of ectoneural-hyponeural interaction, as it does in the radial nerves (Cobb, 1970).

Motor Tracts

The motor tracts that pass radially innervate the lower radial muscles. These muscles lie immediately aboral to the junction region of the circumoral nerve ring and the radial nerve cord. The motor tracts that run inter-radially form the main motor nerve to the substantial external inter-radial muscles. These muscles are innervated by a tract of motor fibres containing about 30 axons from both adjacent radii. The motor tracts from each radius leave the circumoral ring close together at the inter-radial mid point, and there is a single smaller third tract between them. The third tract is very short and forms endings amongst the juxtaligamental cells within the widespread connective tissue which lies between the ambulacral and adambulacral plates; part of the third motor tract also innervates the small internal inter-radial muscle. The layout of motor tracts is illustrated diagrammatically in Figure 23. There are also juxtaligamental cells present where the other motor tracts enter the inter-radial muscles. The motor axons break up into a large number of smaller pre-terminal axons which then innervate the muscles in a similar manner to that described for the intervertebral muscles of the arm.

Basal Lamina

In common with the radial nerve cords, the ectoneural and hyponeural tissues of the circumoral nerve ring are separated by a basal lamina. The basal lamina breaks down at certain points, as it does in the radial nerve cords, and axon bundles pass via these fenestrations to innervate structures that lie oral to the nerve ring.

The processes from muscle tails which are contained in the hyponeural tissue of the radial nerves are also present in the hyponeural tissue of the circumoral nerve ring, although their function remains obscure.

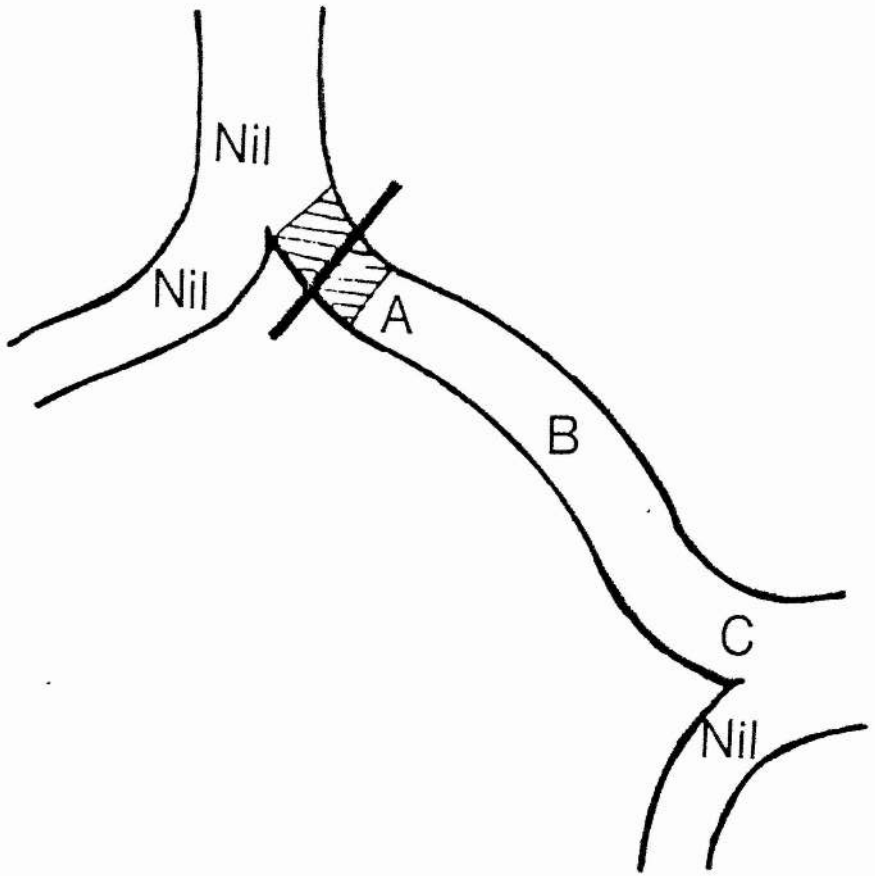
Degeneration Studies

Light microscopic examination of serial sections allowed the layout of the giant fibres to be traced for considerable distances. This method of analysis, although useful, was limited because the giant fibres were always found to end abruptly, apparently without making synaptic contact with other giant fibres. It seems likely that the giant fibres end by breaking up into a plexus of smaller fibres which cannot be traced at the light microscope level. Attempts to trace the axons pathways using cobalt backfilling were also unsuccessful and therefore degeneration studies were used to trace the extent of the giant fibres within the ectoneural tissue.

These studies showed that when the circumoral nerve ring was cut on one side of the junction with the radial nerve (Fig. 30) that Wallerian degeneration occurred in some of the giant ectoneural axons. After four days there was a massive intrusion of coelomocytes and general damage to the nervous tissue in the region immediately adjacent to the cut. At a distance of 1mm from the cut it was possible to count the number of degenerating giant fibres using toluidene blue-stained sections, examined with the light microscope (Fig. 24). The findings under the light microscope were confirmed by examination of ultrathin sections with the electron microscope. Few of the small axons had degenerated at this distance from the cut and there was no increase in the number of degenerating fibres of any size even forty eight days after the cut was made, by which time much regeneration had occurred. Between eighteen and twenty five of the giant axons out of the total of about forty were seen degenerating 1 mm inter-radially from the cut. In the mid inter-radial position an average of ten out of the thirty five giant axons visible at this point showed degeneration, and just before the next radial nerve junction only one or two fibres were seen to have degenerated (Fig. 30). There was no degeneration of the axons in the radial nerve adjacent to where the cut was made, nor in the circumoral nerve ring on the other side of the radial nerve cord. Neither was there any degeneration in the circumoral ring on the side on which the cut was made beyond the next radial nerve junction. The levels of degeneration were consistent between different experimental animals and did not increase between four, seven and ten days. The degenerating axons became surrounded by coelomocytes full of lysosomes and some of the degenerated axons had completely

Fig. 30

Diagram of part of the circumoral nerve ring and two radial nerve cords. The thick line indicates the point at which the nerve cord was severed and the hatched areas (approximately 2 mm either side of the cut) the area that was heavily invaded by coelomocytes. More than half the giant fibres degenerate at A , about ten out thirty five at B and at C only one or two fibres show degeneration. There is no degeneration elsewhere.


Nil
Nil
A
B
C
Nil

disappeared by twenty five days after the cut. Degeneration studies, described earlier in this chapter, have shown a similar situation in the radial nerve cords where individual giant fibres do not extend for large distances. Degeneration studies however do not yield information about how the giant fibres interact synaptically with one another, or with other classes of neurones.

Circumoral Nerve - Radial Nerve Junction

This region consists of a Y-shaped junction with the two parts of the circumoral nerve diverging at an angle of approximately forty degrees from the radial nerve cord, before curving round to the next radius. The giant fibres in the part of the circumoral nerve ring immediately adjacent to the radial nerve cords are arranged circumferentially and there are only small areas of neuropil in these regions. The first segmental ganglion of the radial nerve cords is anatomically similar to the other segmental ganglia except that it is modified to accommodate the convergence of the axons tracts of the circumoral nerve ring (Fig. 31).

Attempts to investigate the anatomy of individual circumoral giant neurones using cobalt backfilling were unsuccessful. The causes of the failure of these experiments are not known. The length of the axons of the giant neurones within the circumoral nerve ring should be great enough to enable successful cobalt backfills to be made.

Fig. 31

Horizontal section through the junction region between the radial nerve cord and the circumoral nerve ring. The layout of this region is similar to the segmental ganglia, with some modification to allow for the divergence of the circumoral nerves.

x 400

Experiments aimed at determining the shapes of individual cells in both the radial nerve cords and the circumoral nerve ring using a modification of Butler's (1971) rapid Golgi method (Keenan et al (1981) were not successful. The protocol used resulted in the deposition of concentric rings of crystals of silver chromate throughout the nerve tissue. Similar results are obtained when Golgi impregnations are made using agarose gel in place of nervous tissue. The formation of these concentric rings of silver chromate is referred to as the Liesgang phenomenon (Stern, 1965). The formation of Liesgang rings may be associated in some way with the lack of glial tissue within the nervous system; when handled the nerve cords lack structural integrity and have a "jelly-like" consistency.

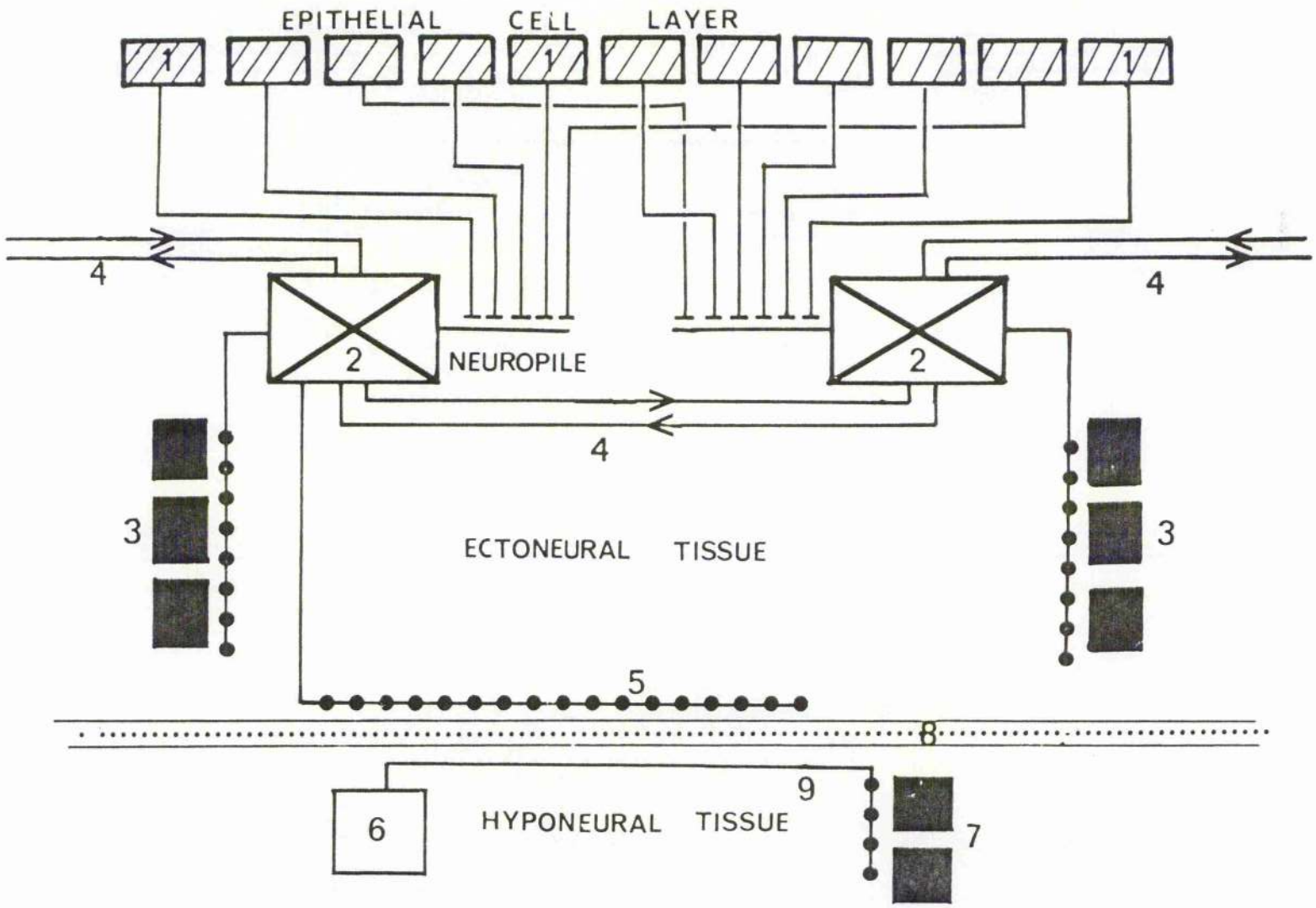
DISCUSSION

Previous attempts to investigate echinoderm neural organisation using asteroids and echinoids have been hampered by the small size of the nerve fibres found in these animals (0.1 μm - 1.5 μm in diameter) which makes the task of tracing the pathways of individual neurones over distances greater than a few microns virtually impossible. A hypothetical model of the echinoderm nervous system has been produced based on the results of ultrastructural studies of starfish nervous systems (Cobb, 1970). Cobb's model (see Fig. 32) proposed the following: Sensory signals from a wide area of epidermis are passed to areas of neuropil within the ectoneural tissue. Motor fibres run from these areas of neuropil to the local effectors, while the areas of neuropil are themselves interconnected by interneurones. There are indirect connections across the basal lamina separating the ectoneural and hyponeural nerves involving primitive forms of synaptic endings; connections are thus effected with the muscles that are innervated by the hyponeural nerves.

The size of the giant fibres within the nervous system of O.texturata has allowed the overall layout of the nervous system to be determined. The results of this study confirm the main tenets of Cobb's hypothesis. The nervous system of O.texturata consists of five radial nerve cords each of which is made up of a chain of interconnected ganglia. The radial nerves are joined by the circumoral nerve ring. There is a massive local sensory input of small fibres to each ganglion which contains extensive areas of

Fig. 32

Diagrammatic representation of a hypothetical model proposed for the echinoderm nervous system based mainly on the results of anatomical studies of asteroids. Sensory cells (1) connect with neurones in the neuropil (2). Motor neurones connect directly with local effectors (3). There are direct interneuronal links (4) between areas of neuropil. There is an indirect link via interneurones and synapses (5) between the hyponeural nerves (6) and the muscles (7) that they innervate. There is some evidence that neuropil occurs also among the hyponeural nerves (9). Basal lamina (8). (From Cobb, 1970)


EPITHELIAL CELL LAYER
1
1
1
4
4
2
NEUROPILE
2
4
3
ECTONEURAL TISSUE
3
5
8
9
6
HYPONEURAL TISSUE
7

neuropil, and a local motor output. The ganglia are joined by tracts of fibres, at least some of which must be interneurones. Each ganglion controls all or part of the effector system in one arm segment and is connected only to the immediately adjacent segmental ganglia and to various peripheral ganglia.

The circumoral nerve ring contains small areas of neuropil that are associated with the innervation of the viscera and skeleton of the disc, but it consists mainly of tracts of axons which connect the adjacent radial nerve cords. It is clear from the results of this study that the radial nerve cords and circumoral nerve ring constitute an unusual form of central nervous system. The echinoderm nervous system was probably highly dispersed when it first evolved, and presumably it still continues to be so in part. The radial nerve cords and circumoral nerve ring can be visualized as having evolved through a concentration and compression of parts of the system into clearly defined tracts, primarily to facilitate communication between areas of neuropil.

The intervertebral muscles of the arms are responsible for locomotion in brittlestars; by suitable combinations of contractions of these muscles the arms can be moved in a variety of directions. The ultrastructural investigation of the innervation of the of the main arm muscles, described in this chapter, was undertaken to determine the manner in which the comparatively small number of hyponeural motor axons innervate the very large number of

muscle fibres that constitute the intervertebral musculature. The basic scheme appears to be simple. The giant axons divide into a number of smaller pre-terminal axons just before or as they enter the muscle block and then each terminal axon innervates many muscle fibres. The large diameter of the motor fibres is undoubtedly an adaptation for a high conduction velocity although other aspects of the motor system do not appear to be adapted for rapid conduction: delays in the transmission of motor information must be introduced by the chemical synapses between the ectoneural and hyponeural tissue and by the small diameter of the fibres in the terminal region of the motor axons. Further delay will be introduced by the small diameter of the fibres from the hyponeural cell bodies, unless these fibres conduct electrotonically.

The innervation of the juxtaligamental cells by the hyponeural nerves was presumed by Wilkie (1979) because of their close anatomical relationship. The present study has examined this problem in detail and has shown from ultrastructural evidence that there is the possibility of both electrical and chemical synaptic contact. The fact that the giant axons and the juxtaligamental cells are closely apposed can only be taken as an indicator that electrical coupling might occur. Even in the best-fixed material the ultrastructural evidence for electrotonic synapses is, on its own, undoubtedly very weak. Much of the difficulty in the identification of electrotonic junctions stems from the difficulty of achieving adequate fixation. Although aldehyde fixation yields good quality preservation of epithelial and muscle tissues it produces rather poor results with nervous tissue. The fixation

technique used in this study was thus a compromise to produce reasonable fixation in all the tissues examined. Freeze fracture and electrophysiological evidence is also required and both these will be practically impossible to achieve in this situation since the areas for study are embedded in blocks of calcite. Electrotonic junctions between cells have not been identified in any adult echinoderm. The presence of pre-synaptic vesicles is the only known form of synaptic specialization found in adult echinoderms (Cobb and Pentreath,1978) and although vesicles are present in the areas where the juxtaligamental cells and the giant motor axons make contact, their presence can only suggest that functional chemical synapses are present. It will require physiological evidence to demonstrate unequivocally the innervation of the juxtaligamental cells by processes from the giant fibres. The function of the juxtaligamental cells that do not contain large granular vesicles is not clear but it seems possible that they are the same cell type as those that contain vesicles but in a different phase of secretory activity.

The innervation of part of the oral intervertebral muscle block by a nerve branch from the hyponeural ganglion in the next arm segment (proximal to the disc) presumably has some functional significance. The part of the muscle block in question has a different anatomical position in relation to the ossicles than the rest of the oral intervertebral muscles and it also makes contact with the longitudinal band of muscle cell processes that penetrate throughout the length of the hyponeural ganglion. The function of these muscle cell processes is obscure, but the ultrastructural

detail is strikingly similar to that of the ampullae in starfish where they have been shown to be involved in neuromuscular transmission (Cobb, 1967). It is therefore possible that some of the oral intervertebral muscles give rise to these muscle cell processes and that they are directly innervated across the basal lamina by fibres of the ectoneural system. The findings of this study are in disagreement with the results reported by Pentreath and Cottrell (1971) from their work on Ophiothrix fragilis. There are two main points of disagreement; Pentreath and Cottrell show the main hyponeural tracts as running distally and aborally away from the ganglion while Hyman (1955), Wilkie (1979) and the present study show them to run proximally and aborally. The muscle cell processes that are described by Pentreath and Cottrell as arising from the intervertebral muscles and their description of the detail of the neuromuscular junction is quite different from that shown by the present study. These authors admit that it was difficult to distinguish nerve from muscle processes in the material they examined and it seems certain that their description is incorrect.

The role of the circumoral nerve ring is thought to be fundamental to the control of behaviour in echinoderms (see Smith, 1966), virtually nothing however is known about its structure. There have been previous accounts of the layout of the circumoral nerve ring in echinoderms (Cuenot,1888; Hamann,1889) but these accounts were both incomplete. The present report is the first attempt to describe the cellular detail of the circumoral nerve

ring in any of the eleutherozoan classes. This work has only been made possible by the large size of some of the nerve fibres found in ophiuroids. This study has shown that the structure of the circumoral nerve ring does not show any great anatomical complexity and is consistent only with providing a functional connection between adjacent radii. All the giant axons run circumferentially and do not show any interweaving with areas of neuropil. The degeneration studies make it clear that none of the axons run for more than one fifth of the circumference of the nerve ring, and that most of the axons do not even run the full distance between adjacent radial nerve cords. The absence of degeneration of the small diameter axons which comprise the bulk of the nerve cord indicates that these fibres run for very short distances, probably only a few tens of microns. The hyponeural axons show no anatomical evidence of involvement in integration; they are bipolar neurones with one process synapsing with the ectoneural nerve fibres and the other directly innervating the muscles.

The basal lamina between the ectoneural and hyponeural tissues, in common with the basal lamina which separates the nervous tissues in the radial nerve cord, is not continuous but contains clearly defined fenestrations. Serial section analysis using both light and electron microscopic sections has shown that these fenestrations are regions where the hyponeural motor axon bundles penetrate the ectoneural tissue to innervate the orally placed muscles and ligaments. It is not clear however, why the basal lamina should break down at these points. In other situations such as the synapses between the ectoneural and

hyponeural tissues in the nerve cords (Cobb,1970 ;Pentreath and Cobb,1972) and the innervation of the tube foot muscles in asteroids (Cobb, 1970; Florey and Cahill,1977, 1980), the basal lamina is continuous between the presynaptic axon and the presumed site of the postsynaptic receptors. The presence of the basal lamina may be a consequence of mechanisms of development in which case it is possible that the ectoneural tissue is derived from ectoderm and the hyponeural from mesoderm. The derivation of nervous tissue from mesoderm would of course be unusual, but it is possible that the hyponeural nerves have evolved from muscle cells. The widespread occurrence of muscle tails that function over long distances as conducting pathways in echinoderms (Pentreath and Cobb,1972) might represent an intermediate stage in the evolutionary process.

Muscles in the tube feet of sea urchins are known to be innervated by nerve endings that are separated from the muscle by a basal lamina, and the functional implications of transmitter release across the basal lamina have been discussed at length (Florey and Cahill,1980). An intracellular investigation of the physiological activities of both types of nerve cell in ophiuroids may shed further light on this problem, as would an ultrastructural investigation of the origins of the nerve tissues at larval metamorphosis.

The innervation of the disc musculature has much in common with the pattern of innervation of the intervertebral muscles of the arm. The radial and inter-radial muscles are innervated by branches from the hyponeural tissue. These nerves are also associated with the juxtaligamental cells, which are thought to be involved in changes in the plasticity of the collagenous ligaments (Wilkie,1979). The anatomical relationship between the the juxtaligamental cells and the muscles and ligaments of the disc is similar to the relationship between the motor nerves and the juxtaligamental cells in the arm. The connective tissue between the first ambulacral and adambulacral ossicles is extensive and it appears to have its own motor branch. Clearly this apparently widespread phenomenon of neurosecretory influence over the state of collagenous connective tissue deserves further investigation.

Although the majority of the musculature associated with the disc is innervated by hyponeural nerves, the muscles of the teeth are innervated by the ectoneural system. This arrangement is similar to the situation in echinoids where, for example, the jaw muscles are innervated by hyponeural nerves and the pedicellarial muscles by ectoneural nerves. One of the unsolved problems in echinoderm nervous systems is the determination of the distribution of sensory and motor fibres in the ectoneural system. There is no evidence as to whether for example, the nerve tracts to the teeth and maxillae contain both motor and sensory fibres or whether separate pathways exist.

Undoubtedly the most important information to arise from the ultrastructural investigation of the circumoral nerve ring is that the arrangement of the neurones in the first segmental ganglia of the radial nerve cord, and in the circumoral nerve ring is surprisingly simple. Where areas of neuropil do occur they are always associated with the departure of minor nerve tracts from the oral nerve ring to innervate parts of the gut and disc. The anatomical evidence, therefore, which still requires physiological confirmation, suggests that the circumoral nerve ring and the radial nerve junction are not integratory centres and that the circumoral ring cannot be considered to be a conventional central nervous system. It has been assumed in a great deal of the recent literature that the circumoral nerve ring does constitute a central nervous system and the present findings will require a re-appraisal of many of the conclusions that have been drawn from the results of past behavioural experiments.

The detailed description of the connections of individual giant fibres in the circumoral ring must await the successful application of intracellular dye-filling techniques. Serial section studies indicate that the giant fibres break up into a number of smaller processes, before making contact with other giant fibres and the mass of smaller neurones. That this can happen is confirmed by the way a few giant hyponeural axons innervate the large number of muscle fibres of the disc and arms . Serial section studies with the electron microscope require an

impracticable number of sections to complete the mapping of the giant cells, and the technique lacks the resolution required to trace all the nerve branches at the light microscope level.

CHAPTER 2

PHYSIOLOGY

INTRODUCTION

Many attempts have been made to analyse the nervous system of echinoderms using electrophysiological techniques, but these have been hampered by the considerable technical difficulties involved in stimulating and recording from the very small, tightly packed nerve fibres. Only a few successful studies have been reported and they have consisted of investigations of the radial nerve cords of starfish and of sea urchins and have revealed summed nervous activity that propagates only short distances and always in a slow decremental fashion (Sandeman, 1965; Millott and Okumura, 1968; Binyon and Hasler,1970; Podol´skii, 1972). The findings of these studies are however not consistent with behavioural observations. For example, the closure of the ambulacral pedicles in starfish and the speed of movement of brittlestars, reveal that information is transmitted rapidly over long distances within the nervous system. Further, simply cutting the radial nerve cords or circumoral nerve ring in starfish or brittlestars interferes with the animals locomotion and coordination. These reports of decrementally conducted activity are of little help in the resolution of such problems as the defining of sensory and motor pathways and determining the role of neuropil. An investigation of the activity of single units within the nerve cords is required for the solution

of these problems.

Unitary potentials have been recorded from the radial nerve cords of an ophiuroid, Ophiopsila californica, in response to electrical stimuli (Brehm, 1977). Brehm's report was the first indication of the possibilities of using ophiuroid preparations for the study of the neurophysiology of echinoderms. The ultrastructural observations on the radial nerve cords of O.californica reported by Brehm, demonstrated the presence of ectoneural axons that were large by echinoderm standards (up to 8 um in diameter). These axons were grouped into bundles of two or three, but there were no more than twelve at any level of cross section of the nerve. Undoubtedly the records of single unit activity obtained from the radial nerve cords of O.californica were records of activity within these large fibres.

Ophiuroids are by far the most active group of present day echinoderms, exhibiting rapid movement in response to photic, tactile and chemical stimuli. This rapid movement suggests that rapid conduction of activity occurs within the nerve cords. Of the species of brittlestar locally available around the coasts of Britain Ophiura texturata is one of the most active; if inverted experimentally it can "right" itself within one or two seconds and moves with great rapidity.

The anatomical investigations described in the last chapter have shown that O.texturata possesses a system of giant fibres within both the ectoneural and hyponeural tissue of the nerve cords. The giant fibres in O.texturata are both greater in size and more numerous than those possessed by O.californica. The extracellular work reported in this chapter shows that the electrical activity from single units within the ectoneural tissue of the nerve cords of O.texturata can be recorded with the aid of suction electrodes. The greater number of units recorded at any one point along the nerve cords in O.texturata compared with the number recorded from O.californica is undoubtedly a reflection of the greater number of giant fibres in O.texturata.

The small size of the majority of echinoderm neurones has hitherto precluded the use of intracellular recording techniques to study individual nerve cells in echinoderms. However the discovery of the giant neurone system in ophiuroids has provided the possibility of carrying out such studies, and intracellular records have been obtained from individual giant cells in both the ectoneural and hyponeural tissues of the radial nerve cords in O.texturata. This is the first report of intracellularly recorded nervous activity in any echinoderm. A range of problems were encountered during the course of attempts to obtain stable intracellular impalements, and much of the work described in this report is thus of a preliminary nature. However, stable impalements can now be obtained with some regularity, and the prospects for future progress are good.

MATERIALS AND METHODS

Extracellular Recording

The experimental animals were initially dissected to reveal the radial nerve cord by removal of the oral arm plates and parts of the lateral arm plates (see Fig. 1 for details of arm structure), followed by removal of the wall of the epineural sinus which overlies the nerve cord. In cases where recordings were to be made from the circumoral nerve ring the nerve ring was exposed by removal of parts of the mouth shield, oral shield and jaw apparatus. Animals thus dissected were immobilised, oral surface uppermost, in a pentaradiate clamp which was subsequently immersed in filtered seawater; in some cases isolated arm preparations were used and in these instances the radial nerve cord was revealed as previously described. The temperature of the preparation was maintained at a predetermined level, usually 10°C, throughout the experiment.

Recordings were made using polythene-tipped suction electrodes with tip diameters between 300 μm and 700 μm. Signals were amplified via high gain AC- coupled amplifiers (Isleworth A103) and were displayed on a Tektronix 561 oscilloscope. Permanent records were made with the aid of a Nihon Khoden oscilloscope camera.

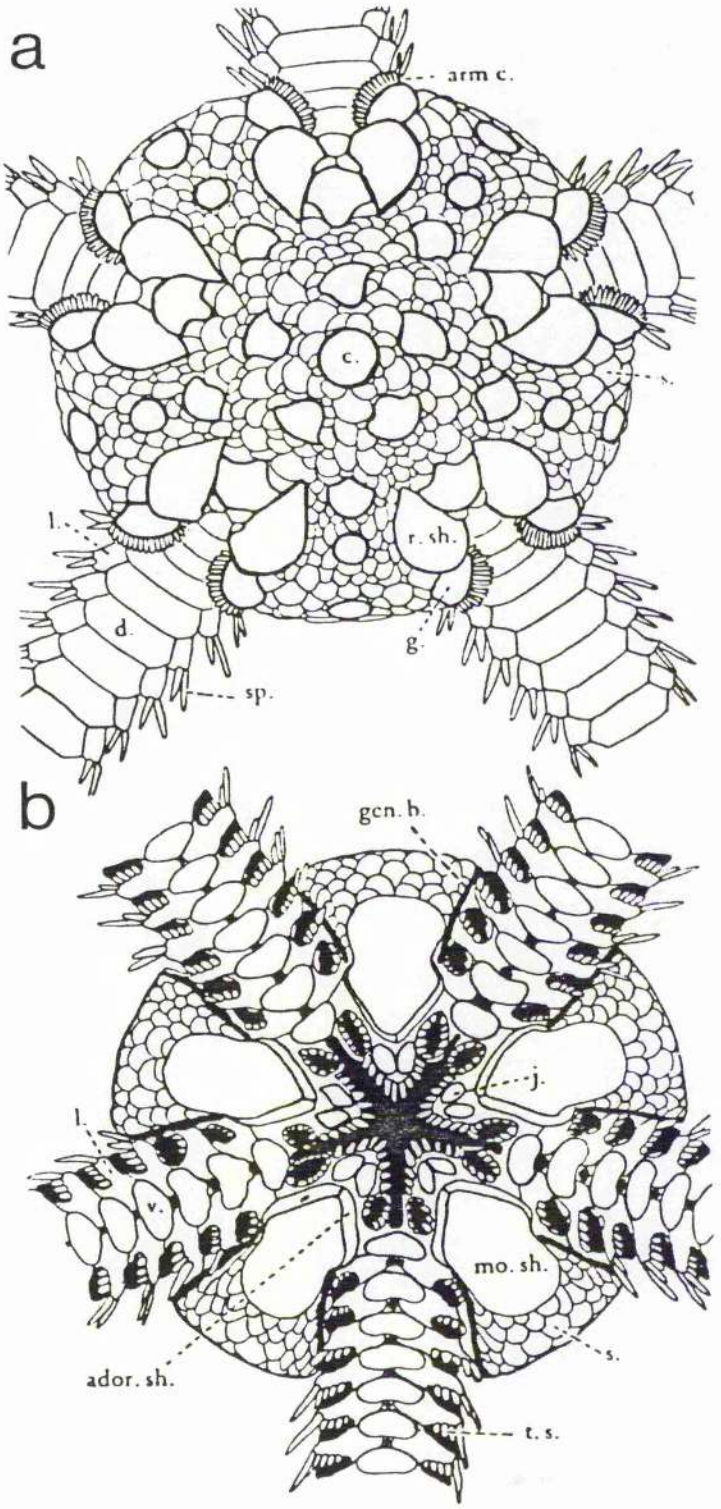
Fig. 1

Ophiura texturata, to illustrate external structure. During dissection the ventral arm plates (v) and parts of the lateral arm plates (l) are removed.

a - aboral surface
b - oral surface

ador. sh. - adoral shield
arm c. - arm comb
c - central plate of disc
d - dorsal arm plate
g - genital plate
gen. b. - genital bursa
j - jaw
l - lateral arm plate
mo. sh. - mouth shield
r. sh. - radial shield
s - scale
sp. - spine
t.s. - tentacle scale
v - ventral plate

(From Eales, 1950)


a
arm c.
c.
r. sh.
l.
d.
g.
sp.
b
gen. b.
j.
l.
v.
mo. sh.
ador. sh.
s.
t. s.

Responses to variety of stimuli were recorded. Electrical stimuli from a Digitimer DS2 isolated stimulator were delivered via suction electrodes; square wave pulses varied in intensity between 0.5 and 10 volts and were of between 50 and 500 msec duration. Chemical stimuli were applied in solution in seawater, via a syringe. Mechanical stimuli were provided by touching the arms, disc and tubefeet with a thin glass rod. Experiments involving other than photic stimulation were carried out under constant lighting conditions to prevent accidental photic stimulation of the experimental animal.

Photic stimuli were found to be the most reliable way to elicit a recordable response within the nerve cords. The light source was a plane filament tungsten lamp run at 2800^{o}K from a stabilized 6v DC supply. Light stimuli were transmitted to the preparation via a 2 mm diameter fibre optic cable (Barr and Stroud Ltd.) to which a number of apertures could be fitted to give a light spot of between 0.5 and 5 mm in diameter. A series of interference filters with emisson peaks ranging from 430 to 610 nm were used in conjunction with appropriate neutral density filters, to determine the action spectrum of the photic response. In all other experiments a 482 nm interference filter and a neutral density filter were inserted into the light path. The light source was calibrated with aid of an EMI 9871R photomultiplier tube. Light stimuli delivered to the preparation were controlled by a camera shutter inserted into the light path and operated via a cable release. Indication of light "ON"/"OFF" was given by a

photoconductive cell also inserted into the light path. All extracellular experiments involving photic stimulation were carried out in the dark.

By virtue of the fact that the radial nerve cord was exposed by dissection from the oral surface all the extracellular recordings from the radial nerves were obtained from the ectoneural part of the nerve cord. Recordings were also made from animals restrained in the more usual "aboral surface uppermost" position, using inverted suction electrodes (see Fig. 14). In addition recordings were made from the radial nerve cords whilst the experimental animal was inverted in a simulation of the "righting" response. Fig. 2 illustrates the apparatus used for this experiment.

Attempts were made to obtain extracellular recordings using a variety of types of electrodes, other than suction electrodes. These included the use of silver wire hook electrodes and plastic insulated tungsten electrodes. Records were only obtained when suction electrodes were used.

Intracellular Recording

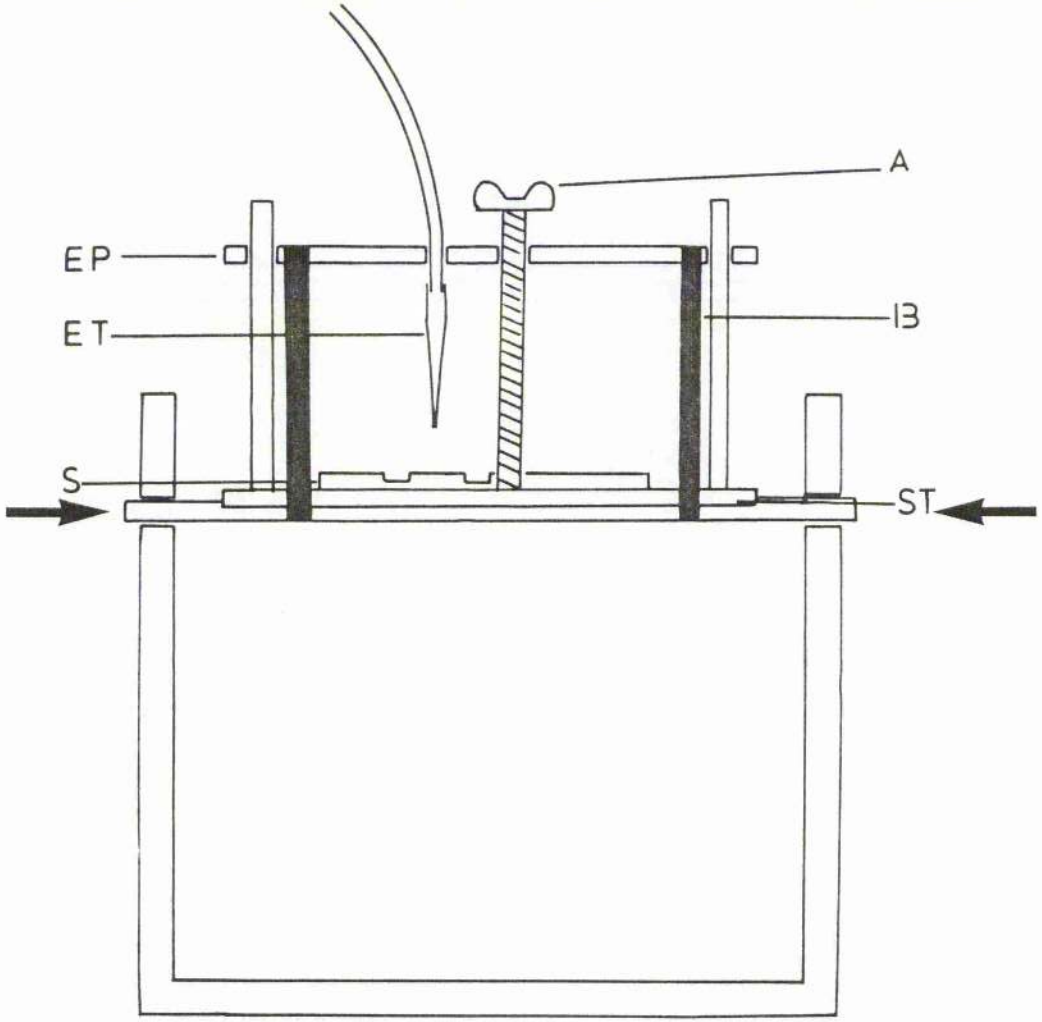
Three different methods of preparing the radial nerves for intracellular experiments were employed.

1) The radial nerve cord was exposed by removal of the oral

Fig. 2

Diagram of the apparatus that was used to record from the radial nerve cord whilst the experimental animal was inverted in a simulation of the "righting response". This apparatus was also used to record responses to photic stimulation in inverted and in non-inverted animals. The animal is restrained in the specimen clamp (S) which is held in position on the rotating stage (ST) with the aid of petroleum jelly, and is positioned directly under the electrode (ET). The electrode is lowered by means of the adjustment screw (A) which allows the electrode plate (EP) to move up or down against the force of the elastic bands (B). The apparatus is immersed in a tank of seawater and is rotated about its' axis (arrowed).

Not to scale


A
EP
ET
IB
S
ST

arm plates and parts of the lateral arm plates. The arm was subsequently severed from the disc. The nerve cord was then stained in situ for five minutes in a dilute solution of neutral red in seawater, to render the nerve cord clearly visible and thus to facilitate dissection. After staining, the nerve cord was dissected free from the ossicles and was pinned out with the aid of cactus spines in a sylgard-lined petri dish containing approximately 50 mls of chilled, filtered seawater. In the intact arm the radial nerve cord is covered by the walls of the epineural sinus. The oral wall of this sinus was inevitably removed during the dissection of the radial nerve cord from the arm. The aboral sinus wall however remained closely applied to the surface of the nerve cord and therefore had to be removed prior to recording. Failure to remove this second wall prevented successful recording.

2) The nerve cord was exposed and stained by the method described in (1). The severed arm, with the radial nerve cord in situ, was then immobilised in a clamp which was subsequently immersed in chilled, filtered seawater. Both the oral and aboral walls of the epineural sinus were removed prior to recording.

3) The hyponeural part of the radial nerve cord was revealed by cutting through the arm so that the aboral arm plates, much of the lateral arm plates and most of intervertebral ossicles with their associated musculature were removed, leaving the walls of the hyponeural sinus, the nerve cord and the oral arm plates intact. The hyponeural tissue was then accessible via the remains of the hyponeural sinus. The remainder of the arm thus dissected was

stained with neutral red and after rinsing with fresh seawater, was pinned out in a sylgard lined petri dish and covered with chilled, filtered seawater. In this case the wall of the hyponeural sinus had to be removed prior to recording.

The use of neutral red rendered the nerve cord clearly visible and extracellular recordings from stained radial nerve cords demonstrated that the function of the nervous system was unimpaired by the staining process. In cases where nerve cords were stained with neutral red for periods of up to two hours, small groups of cell bodies in both the ectoneural and hyponeural tissues stained far more intensely than did the rest of the nerve cord (Fig. 3). It is possible that these cells contain catecholamine or 5-hydroxytryptamine, as do cells that stain intensely with neutral red in leech ganglia (Stuart et al., 1974).

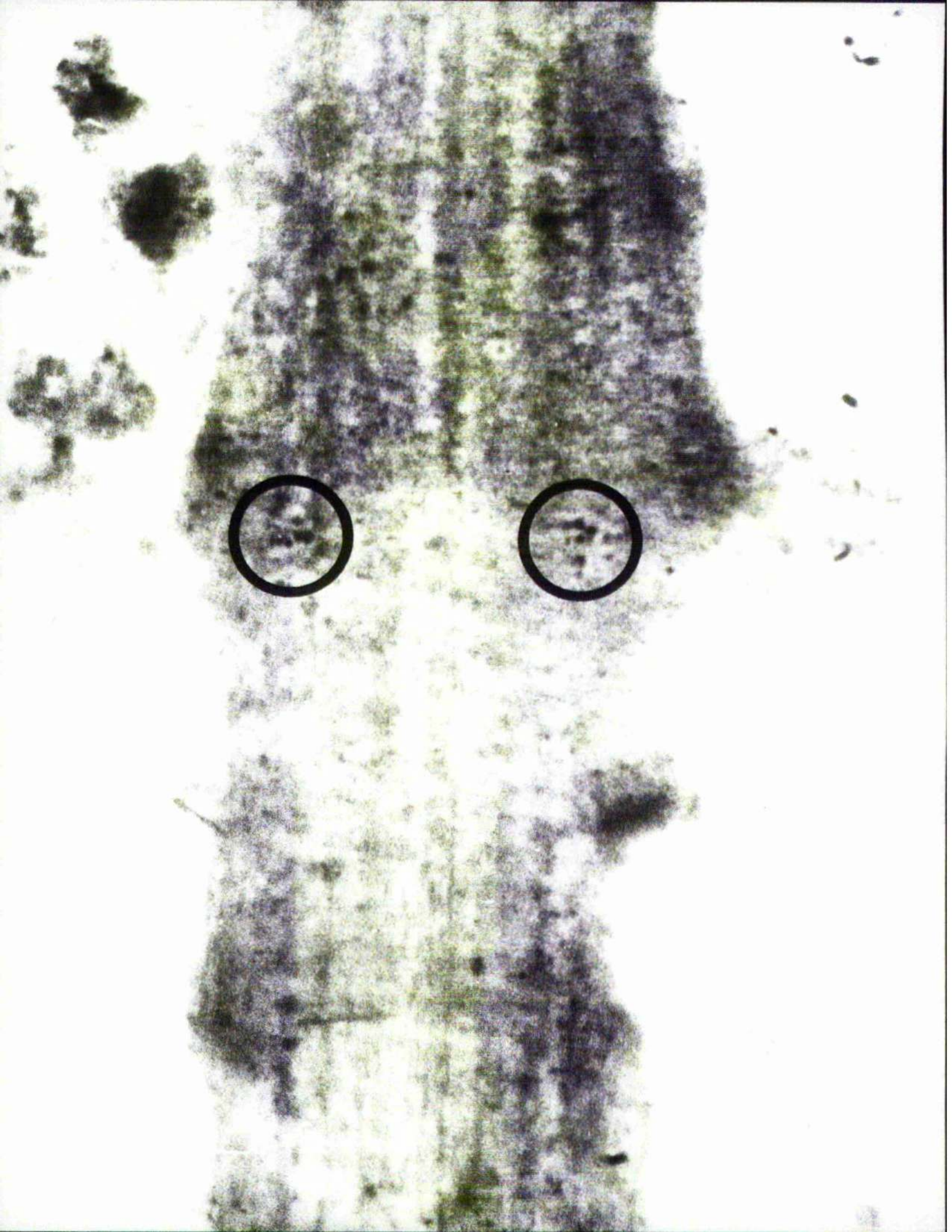
Throughout all intracellular experiments the temperature of the preparation was maintained at $10^{\circ}C \pm 1^{\circ}C$

Recordings were made using glass microelectrodes pulled from 1.5 mm fibre-filled thick-walled capillary glass (Clark Electromedical GC150-F) using an Industrial Science Associates electrode puller. When filled with 2M potassium chloride these electrodes had tip resistances of between 80 MΩ and 150 MΩ . In some experiments electrodes were pulled from 1,5 mm thin-walled glass, such electrodes had resistance values between 30 MΩ and 60 MΩ when filled with 2 M potassium chloride. Signals were amplified via a low gain D.C. amplifier and were displayed on a

Fig. 3

Small groups of cell bodies in both the ectoneural and hyponeural tissue stain more intensely than the rest of the tissue with neutral red. In this case the two groups of ectoneural cell bodies which stain in each segment are shown (ringed).

x 100



Tektronix 561 oscilloscope. Permanent records were obtained with a Nihon Khoden oscilloscope camera. Electrodes filled with Lucifer Yellow CH (Stewart, 1978) and backfilled with 1M lithium chloride were used for recording and dye-filling experiments. Electrodes filled with 100 mM cobalt chloride or 100 mM nickel chloride (modified from Delcomyn, 1981) were also used for dye-filling experiments. Hyperpolarizing 10 nA current pulses of 1 second duration at a frequency of 1 Hz were used to inject Lucifer. Cobalt and nickel chloride were injected by passing depolarizing 5 nA current pulses of 1 second duration at a frequency of 1 Hz, via the tip of the electrode. Preparations containing cells that had been injected with Lucifer were fixed in 4% formalin in seawater, dehydrated in alcohol and subsequently cleared in methyl salicylate. Wholemount preparations were viewed with the aid of a Zeiss transmission fluorescence microscope with a Zeiss BG 12 excitation filter and Zeiss 44 and 53 barrier filters. Cobalt and nickel filled preparations were processed by the addition of 2 or 3 drops of ammonium sulphide to the surrounding seawater. After approximately 10 mins the preparation was fixed in 4% formalin in seawater, dehydrated in alcohol and cleared in methyl salicylate. Preparations were then examined with the light microscope.

In some cases thick-walled electrodes were beveled using either a jet stream beveler (Ogden and Citron, 1978) or a modification of the jet stream beveler (Corson et al., 1979). In either case electrodes were beveled until their resistance had fallen to between 30 MΩ and 60 MΩ (for thick-walled electrodes filled with 2 M potassium chloride).

Advance of the microelectrode was achieved with the aid of a hydraulic advance system (Clark Electromedical HMD-1) mounted on a Zeiss (Jena) sliding plate manipulator. In some experiments a piezoelectric drive unit (Chen, 1978) was used in an attempt to achieve good impalements.

RESULTS

Extracellular Recordings

The mechanical stimulation involved in the initial attachment of the suction electrodes to the nerve cords resulted in a burst of single unit activity which was allowed to die down before stimulation and recording were begun. Continuous monitoring of the activity within the radial nerve cords of an immobilised animal demonstrated the presence of bursts of spontaneous activity (Fig. 4). During some such bursts pronounced movements of the tubefeet were noted.

Recordable single unit activity within the nerve cords could be elicited by chemical, electrical, mechanical and photic stimuli; of these photic stimulation was found to be the most reliable way of eliciting a response.

1) Responses to Photic Stimulation

Stimulation of the oral surface of the distal portion of an arm, with a light spot 2 mm in diameter resulted in recordable activity within the nervous system. A complex train of single unit spikes, lasting for up to twenty five seconds, was propagated along the nerve cord in response to a light "OFF" stimulus (Fig. 5); a less marked response to light "ON" could also be recorded but contained fewer units and was of shorter duration (Fig. 6). The recorded potentials had a spectrum of amplitudes up to 80 μV with

Fig. 4

Bursts of spontaneous activity recorded from the radial nerve cords of O.texturata.

Time calibration = 1 sec

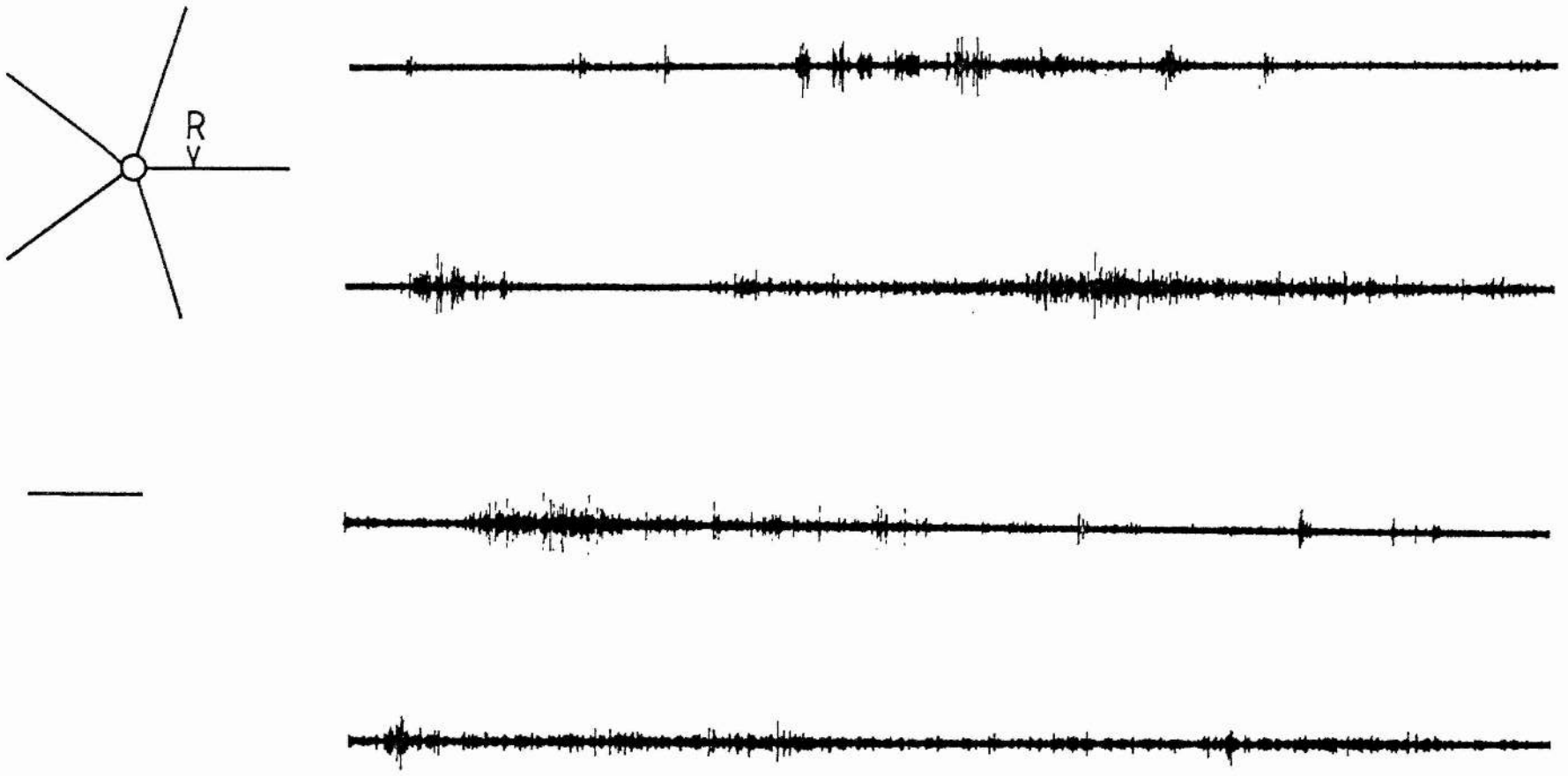

R
Y

Fig. 5

A response to a light "OFF" stimulus. The deflection in the stimulus trace indicates the point at which the stimulus was applied. The direction of the deflection is not significant. Continuous records.

Time calibration = 1 sec

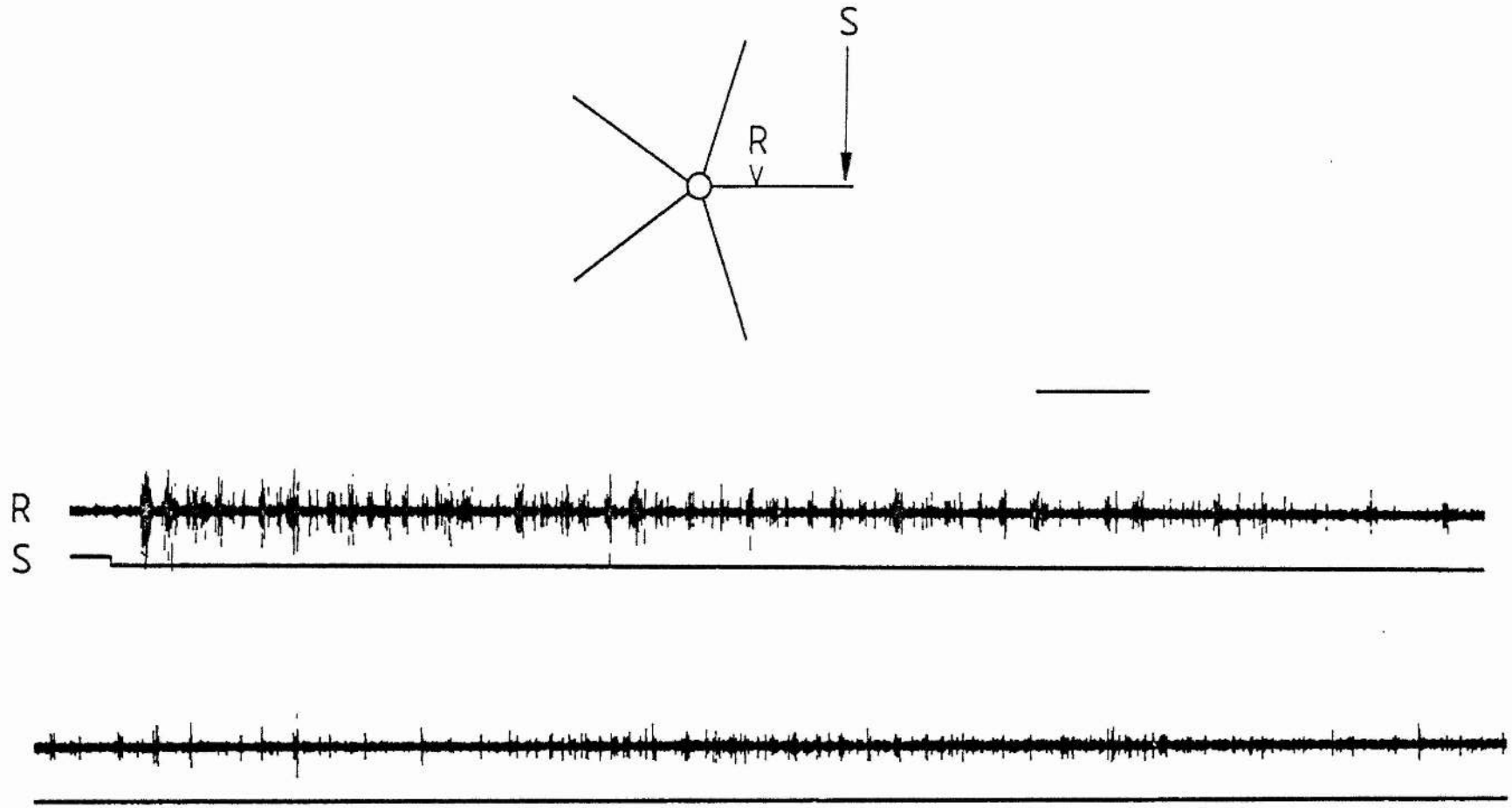

S
R
R
S

Fig. 6

A response to light "ON". Responses to light "ON" are of shorter duration and contain fewer units than do responses to light "OFF".

Time calibration = 1 sec

S
R

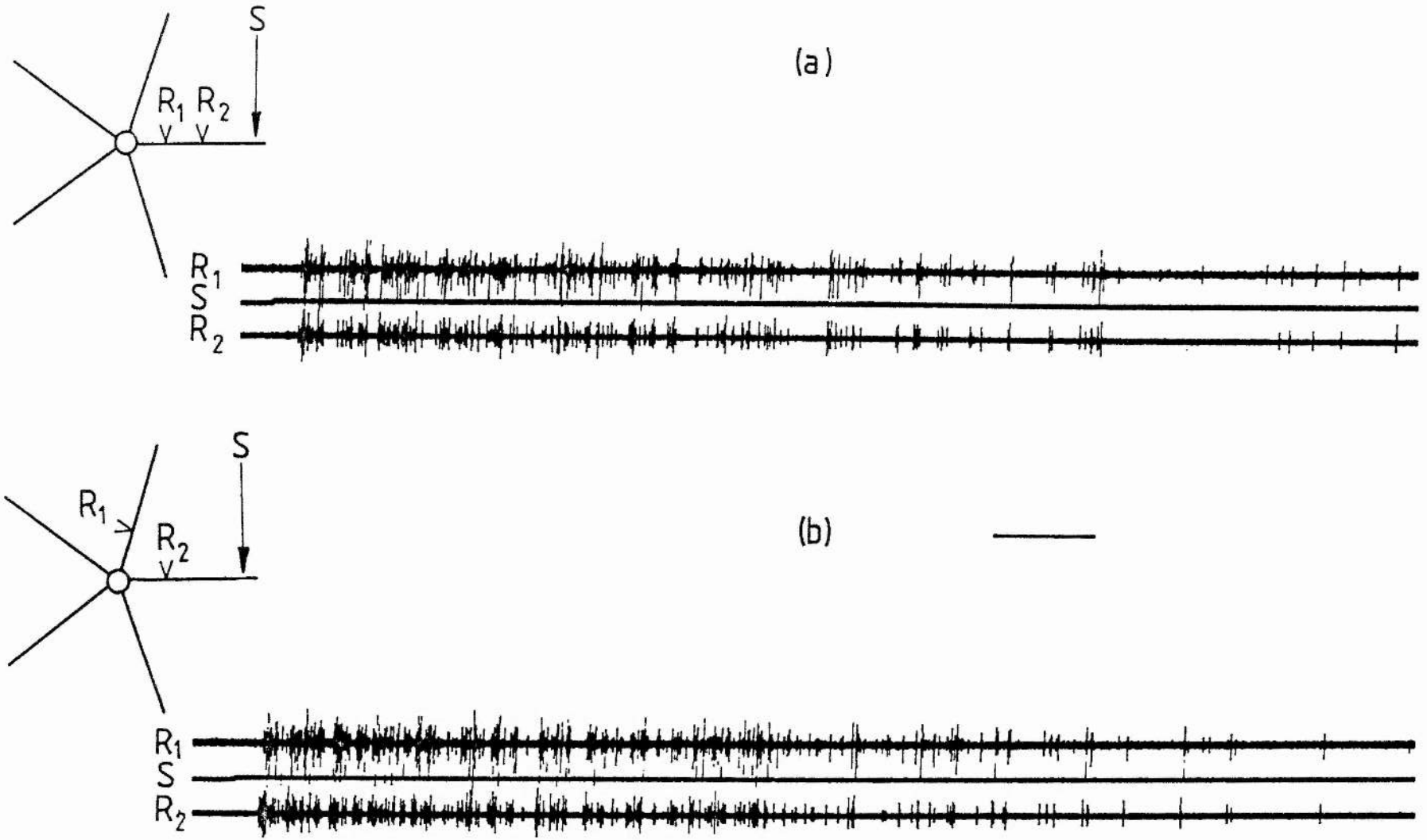
R
S

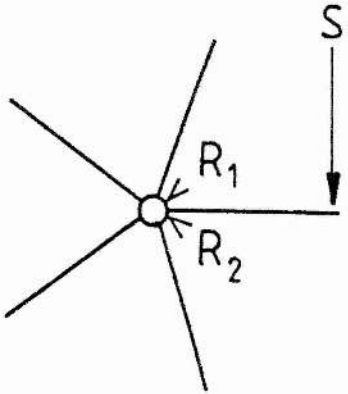
individual durations ranging from 10 to 20 msec. Values for the conduction velocity of these spikes were calculated from the time delay in the arrival of the leading spike at two recording electrodes, which were separated by a known distance. Values thus derived ranged between 400 and 950 mm/s, with an average value of 800 mm/s. Records obtained using electrodes attached to the radial nerve cord in the stimulated arm, and to the radial nerve cords in the other arms, and to the circumoral nerve ring showed that the light response was conducted along the radial nerve cord in the stimulated arm, and thence to all other arms via the circumoral ring (Fig. 7). Cutting the circumoral nerve ring to one side, or to the other, or on both sides, of the stimulated arm demonstrated unequivocally that the spike train was conducted in both directions around the circumoral nerve ring (Fig. 8). Records obtained from the radial nerve cords of different arms in response to photic stimulation showed a similarity both in the number of units involved and in the duration of the response, indicating that the response was conducted to all other parts of the animal in a substantially unaltered form (Fig. 9). However, in the stimulated arm there were differences between the centrifugally conducted (away from the disc) and the centripetally conducted (toward the disc) responses (Fig. 10); the centrifugally conducted responses contained fewer units and were of shorter duration. The response to light "OFF" was made up of a series of bursts of activity at approximately 5 Hz (see Fig. 5). The varying sizes of the potentials in each burst make it clear that several units are involved in each. The bursts may have been derived in one of two ways: they may have been the result of several cells being driven

Fig. 7

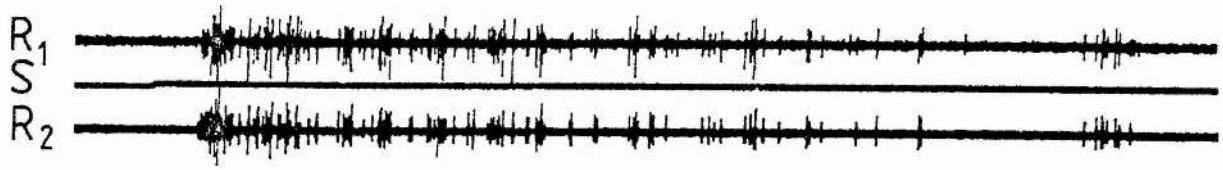
The response to a photic stimulus is conducted along the radial nerve cord in the stimulated arm (a) and thence to the other arms (b) via the circumoral ring (c) and (d).

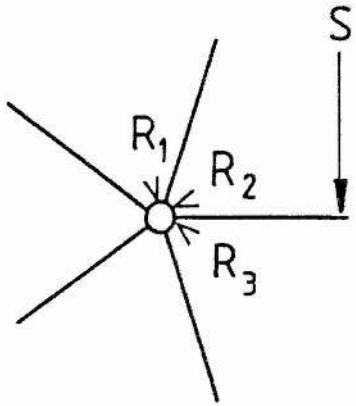
Time calibration = 1 sec


S
R1
R2
(a)
R1
S
R2
S
R1
R2
(b)
R1
S
R2



(c)





(d)

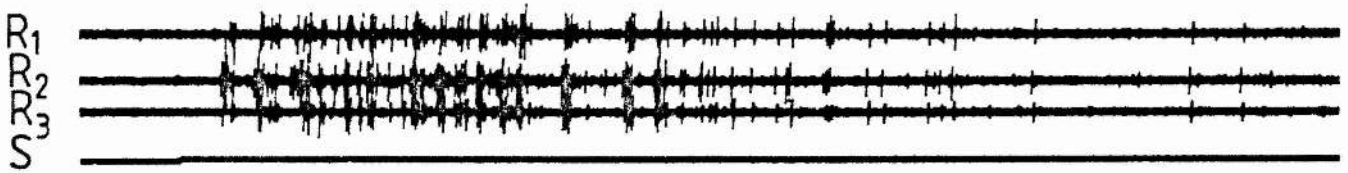
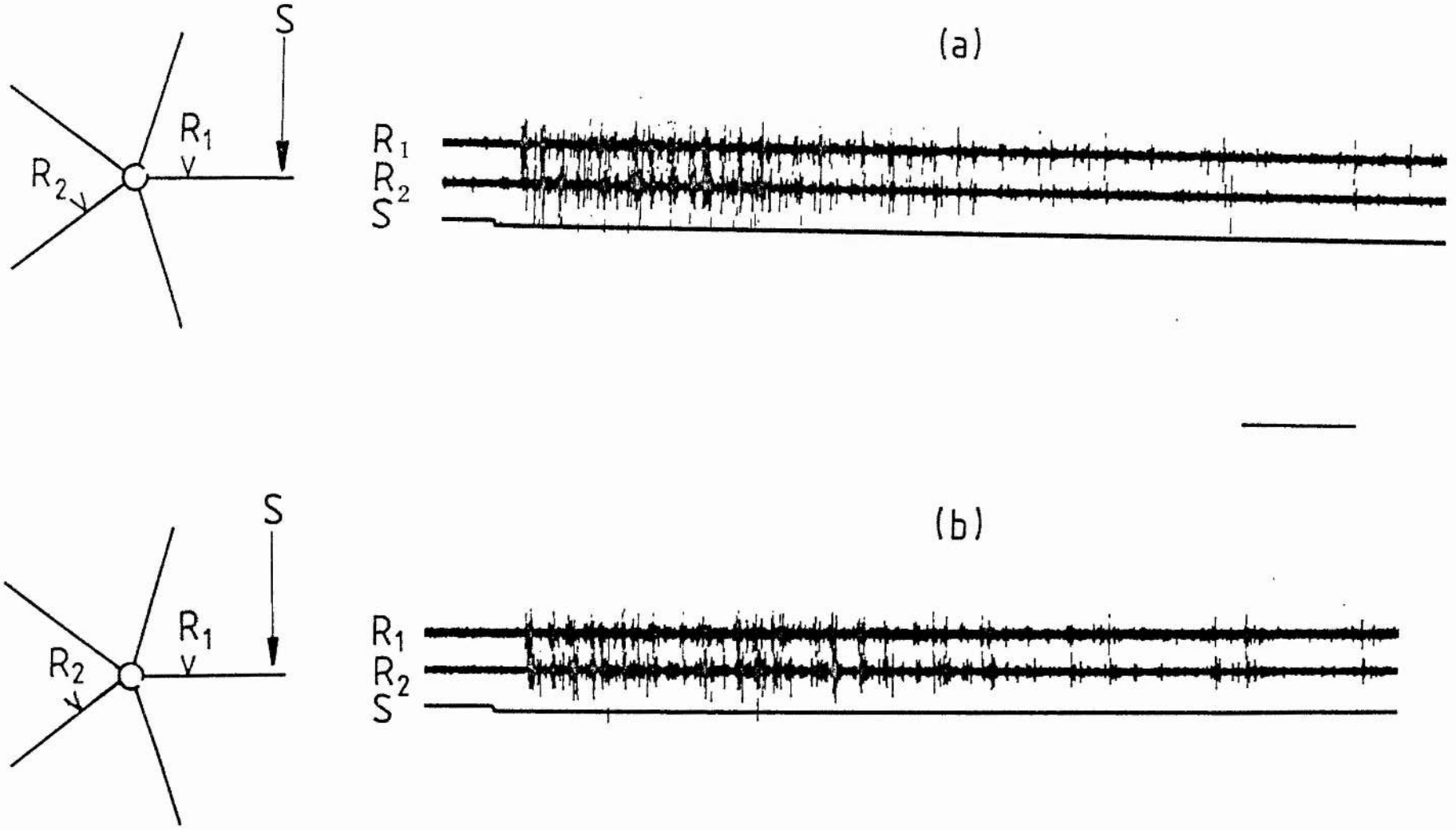


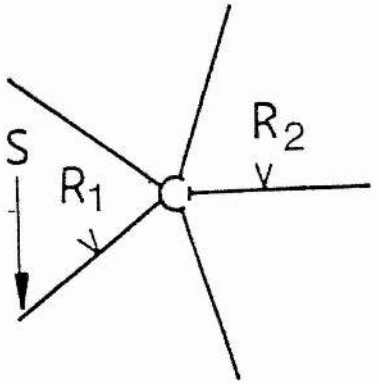
Fig. 8

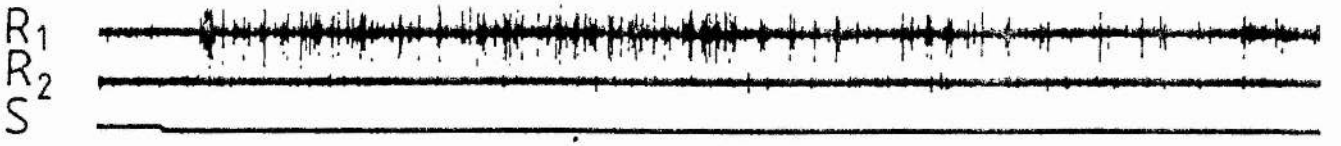
Activity elicited in response to photic stimulation is conducted around the circumoral ring in both directions. Cutting the circumoral nerve ring to one side (a) or the other (b) of the stimulated arm does not prevent conduction to the other arms. Cutting the nerve ring on both sides of the stimulated arm prevents conduction to the other arms. To check that R2 had not become detached from the nerve cord the arm was mechanically stimulated, resulting in a response (d).

Time calibration = 1 sec

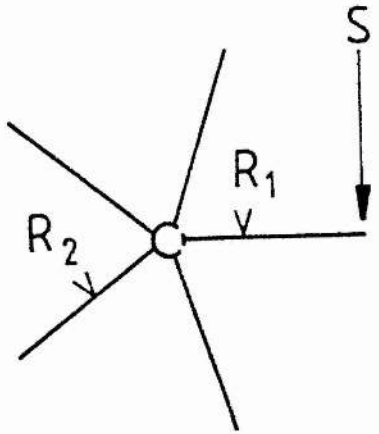

S
R1
R2
(a)
R1
R2
S
S
R1
R2
(b)
R1
R2
S

(c)





(d)



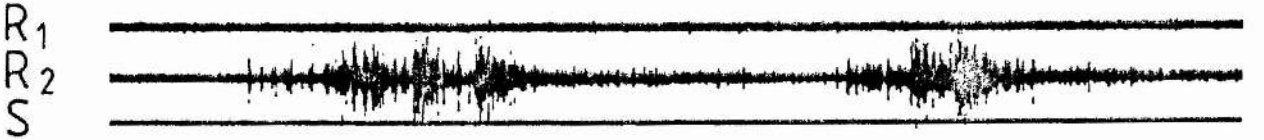
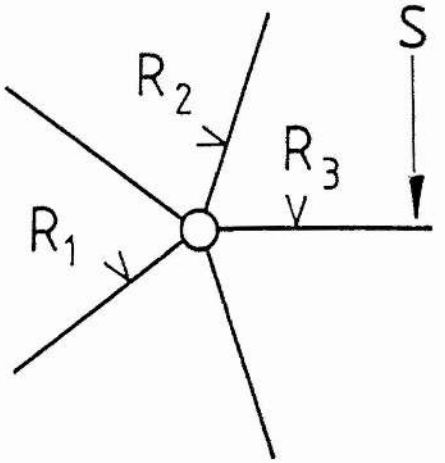


Fig. 9

Responses to photic stimuli are conducted to all other arms in a substantially unaltered form. The arrows indicate groups of units detected at all three recording sites.

Time calibration = 1 sec


S
R2
R3
R1

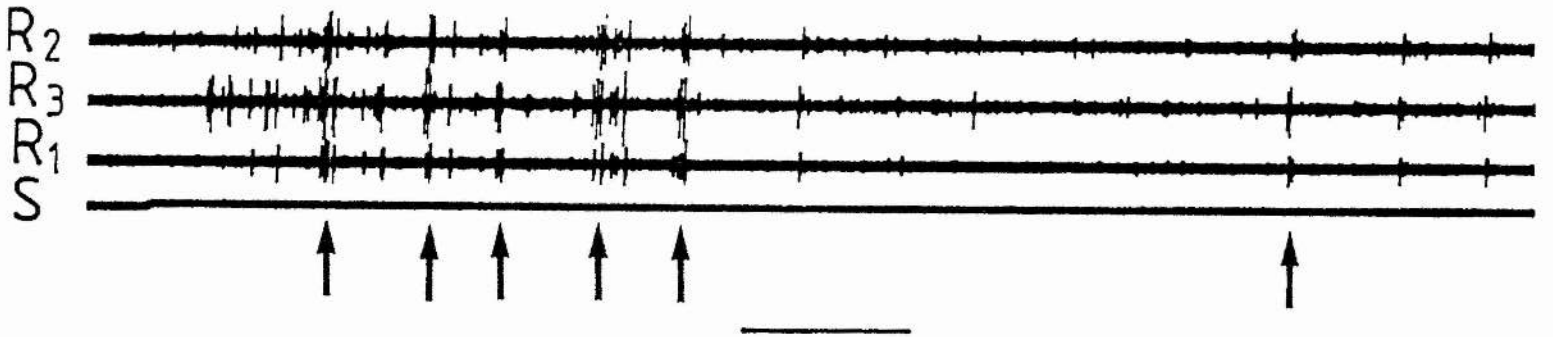
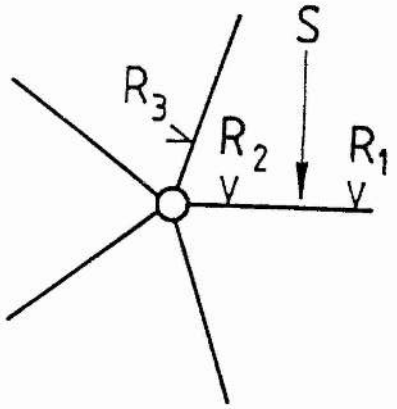
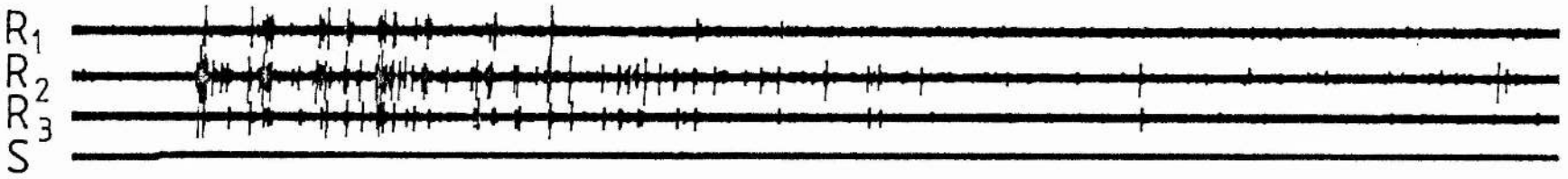

R2
R3
R1
S

Fig. 10

There are differences between centrifugally conducted responses and centripetally conducted responses in the stimulated arm.

Time calibration = 1sec


S
R3
R2
R1

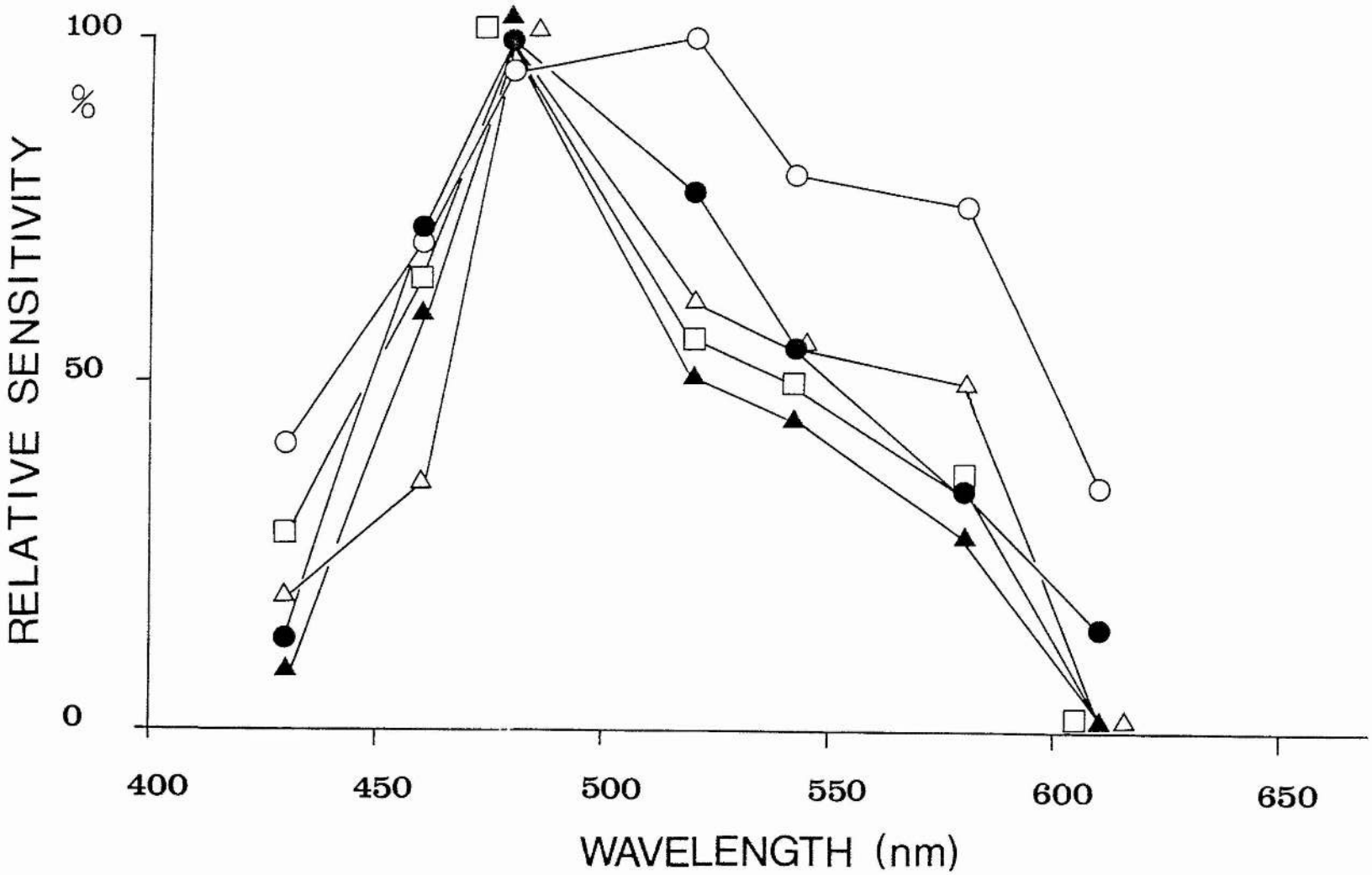

R1
R2
R3
S

by a common input from a single cell firing at 5 Hz, or they may have been produced by spike discharge at 5 Hz in a number of cells that were synaptically coupled, and thus phase-locked.

The action spectrum of the photic response was determined using light stimuli of known wavelengths. The response was quantified by counting the total number of potentials recorded during the response, using records filmed at high speed, and assigning an arbitrary value of 100% to the maximal response. The average response duration was 14 secs, however it was possible that in some cases the duration of the apparent response was increased by co-incident spontaneous activity. Ten minutes was allowed between each stimulation in an effort to minimize effects due to habituation to the light stimuli. Fig. 11 illustrates the action spectrum of the response thus determined for five individuals and indicates that the maximal response was elicited by light at the blue/green end of the spectrum. The light source was calibrated with the aid of the photomultiplier tube to an energy value of 10^{-5} uW $/m^2$; this value falls within the range of light levels encountered by animals living in clear coastal waters (Clarke and Denton,1962). Illumination of the arm for periods of 10 to 15 secs at this intensity was sufficient to elicit a response to light "OFF"; a response to light "ON" at this intensity was obtained only after the animal had been dark adapted for at least five minutes.

Fig. 11

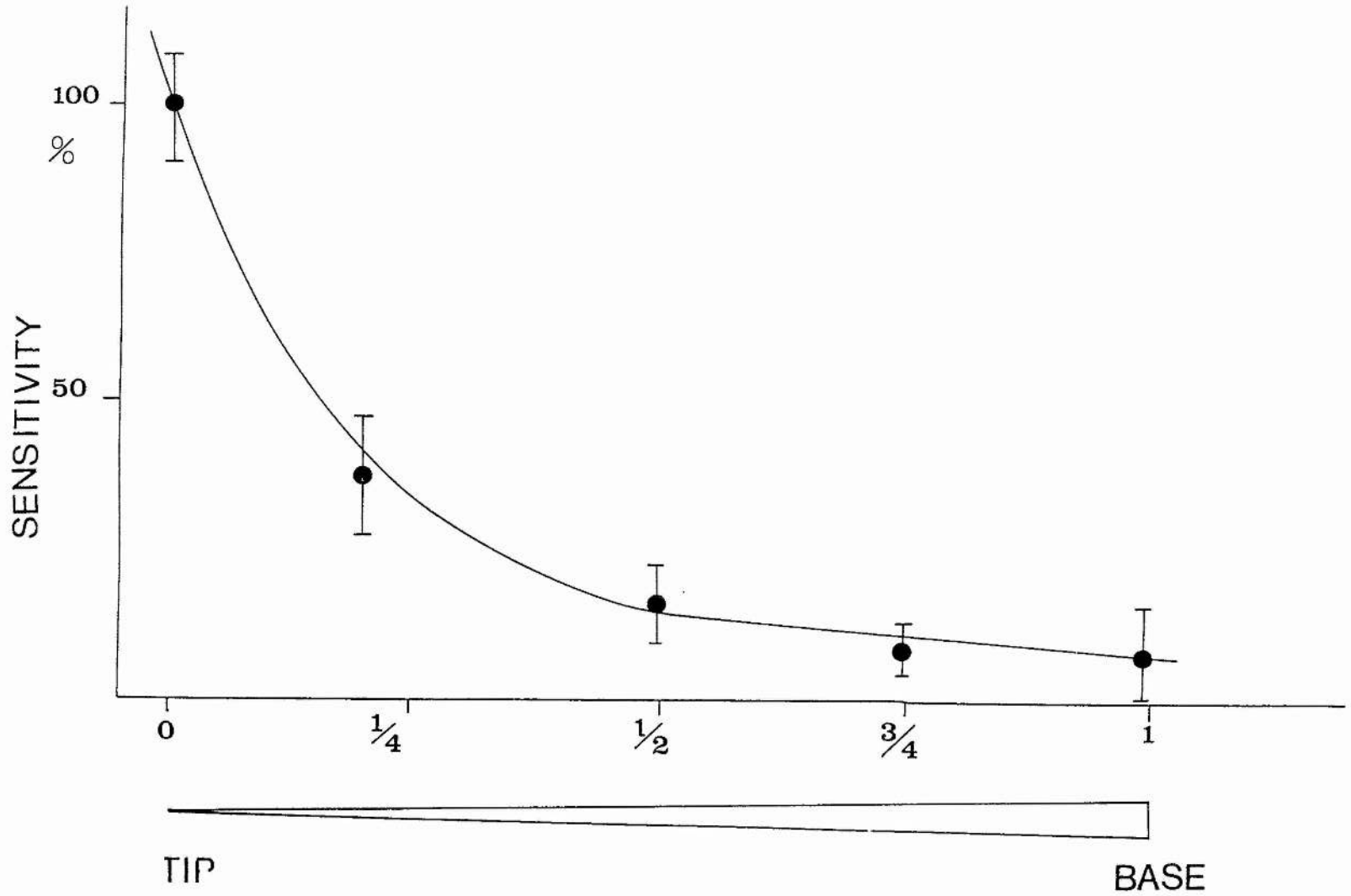
A graph illustrating the action spectrum of the light response from five individuals. Each response was quantified by counting the total number of potentials recorded. The maximal response is elicited by light at the blue/green end of the spectrum.


100
%
50
0
RELATIVE SENSITIVITY
400
450
500
550
600
650
WAVELENGTH (nm)

The sensitivity of an arm to a stimulus of a given energy was found to vary inversely with the distance away from the arm tip, in some cases the arm tip was found to be an order of magnitude more sensitive than the arm base. Responses were again quantified by counting the total number of potentials in a given response; an arbitrary value of 100% being assigned to the maximal response (see Fig. 12). Ten minutes was allowed between each stimulation in an effort to minimize any effects due to habituation of the response. Stimulation of an arm by light spots of varying diameters with the intensity adjusted to a uniform level using neutral density filters established that irrespective of the position of the light spot on the stimulated arm, a response was obtained only when more than one segment of the arm was photically excited. Variations in the intensity of the light stimuli resulted in an alteration of the spike frequency and response duration. The experimental animals were found to react to light stimuli of intensities outside the normal physiological range (i.e. fluorescent room lights etc.). Responses to light "OFF" were not recorded from isolated radial nerve cord preparations or when the oral and lateral arm plates had been dissected away from the entire length of the stimulated arm. This suggests that the nerve cells making up the radial nerves were not themselves photosensitive and that the photoreceptive structures were located peripherally, probably within the dermal plates. These results contrast with those reported for isolated radial nerve cord preparations of Diadema setosum where the neural elements themselves were thought to be light sensitive (Takahashi,1964).

Fig. 12

The sensitivity of the arm to a given light stimulus was found to vary inversely with the distance away from the arm tip. The response was quantified by counting the total number of potentials in a given response. An arbitrary value of 100% being assigned to the maximal response. The position of the light spot along the arm is expressed as a fraction of the total arm length, in the arm base to arm tip direction. This graph represents the data from five individuals.


100
%
50
SENSITIVITY
0
1/4
1/2
3/4
1
TIP
BASE

Ultrastructural examination of decalcified material, prepared by methods described in chapter 1, with the light and electron microscopes revealed none of the structures usually associated with well defined photoreceptors (for a review of invertebrate photoreceptor structure see Eakin,1972). Photosensitivity in other echinoderms which also lack well defined photoreceptors has been referred to as a "dermal light sense" (Millott, 1975).

The results described above were all obtained from specimens of Ophiura texturata but responses to light "OFF" have also been recorded from the radial nerve cords of specimens of Ophiothrix fragilis and of Ophiocomina nigra (Fig. 13). The responses recorded from specimens of these two species were however less marked than the responses recorded from O.texturata, reflecting the greater numbers and size of the giant fibres in the latter.

Experiments involving animals restrained in an inverted clamp (with the animal in the more natural "oral side down" position) and using inverted suction electrodes (Fig. 14) demonstrated similar responses in inverted and in non-inverted animals (Fig. 15). In these cases the same preparation could not be used for both inverted and non-inverted experiments because of damage sustained by the nerve cord during detachment of the electrodes; the nature of this damage is discussed at greater length later in this chapter. However, responses to light "OFF" in a single preparation were obtained using the apparatus designed for an investigation of the righting response (see Fig. 2 for diagram of apparatus).

Fig. 13

Responses to light "OFF" recorded from other ophiuroids.
a) Ophiothrix fragilis
b) Ophiocomina nigra
These responses to light "OFF" are less marked than those recorded from O.texturata, and this probably a reflection of the greater number and size of the giant neurones in O.texturata.

Time calibration = 1 sec

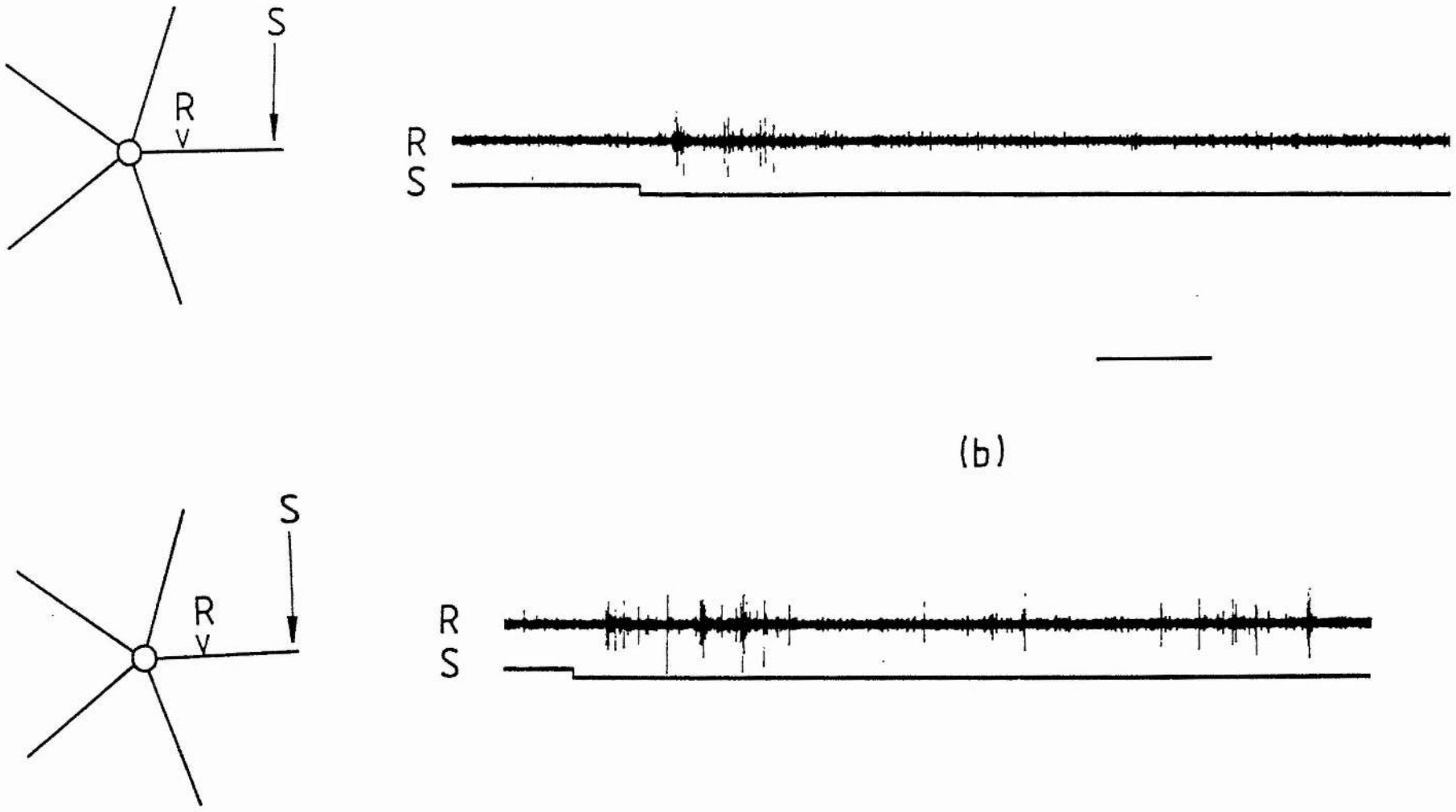

(a)
S
R
R
S
(b)
S
R
R
S

Fig. 14

Diagram of the apparatus used to record from animals held in the more natural "oral side down" position. The animal is restrained in the specimen clamp (SC) and the apparatus is immersed in a tank of seawater. The inverted suction electrode (R) is positioned with aid of the mirror (M). Light stimuli can be supplied to the preparation via the fibre optic light guide (L), which may be used to stimulate either the oral or the aboral surface of the animal.

Not to scale

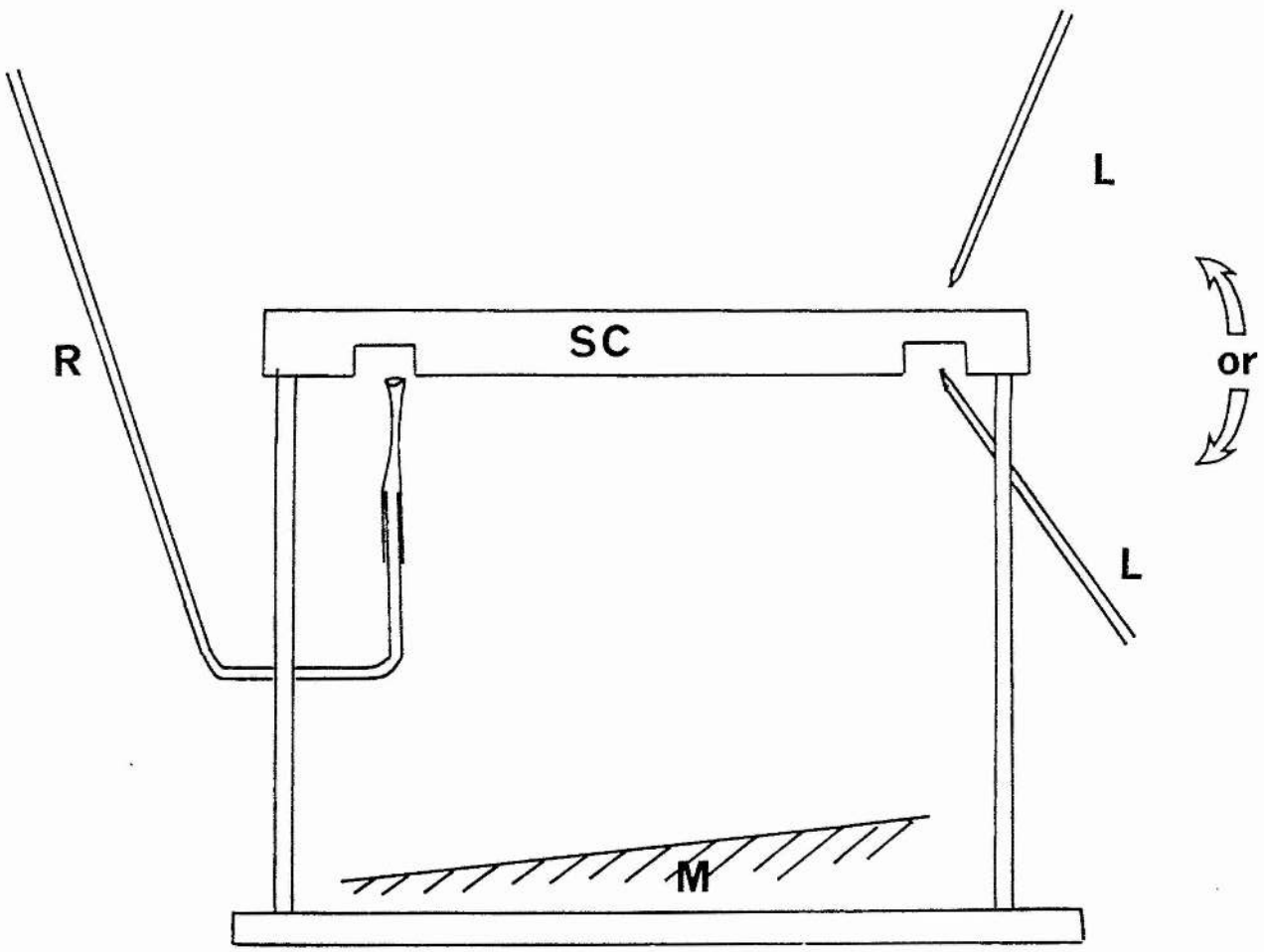

L
R
SC
or
L
M

Fig. 15

Activity recorded using inverted suction electrodes and animals restrained in the more natural "oral surface down" position were similar to those recorded from animals restrained with the oral surface uppermost.
a) A response to light "OFF" recorded using inverted electrodes.
b) Spontaneous activity recorded using inverted electrodes.

Time calibration = 1 sec

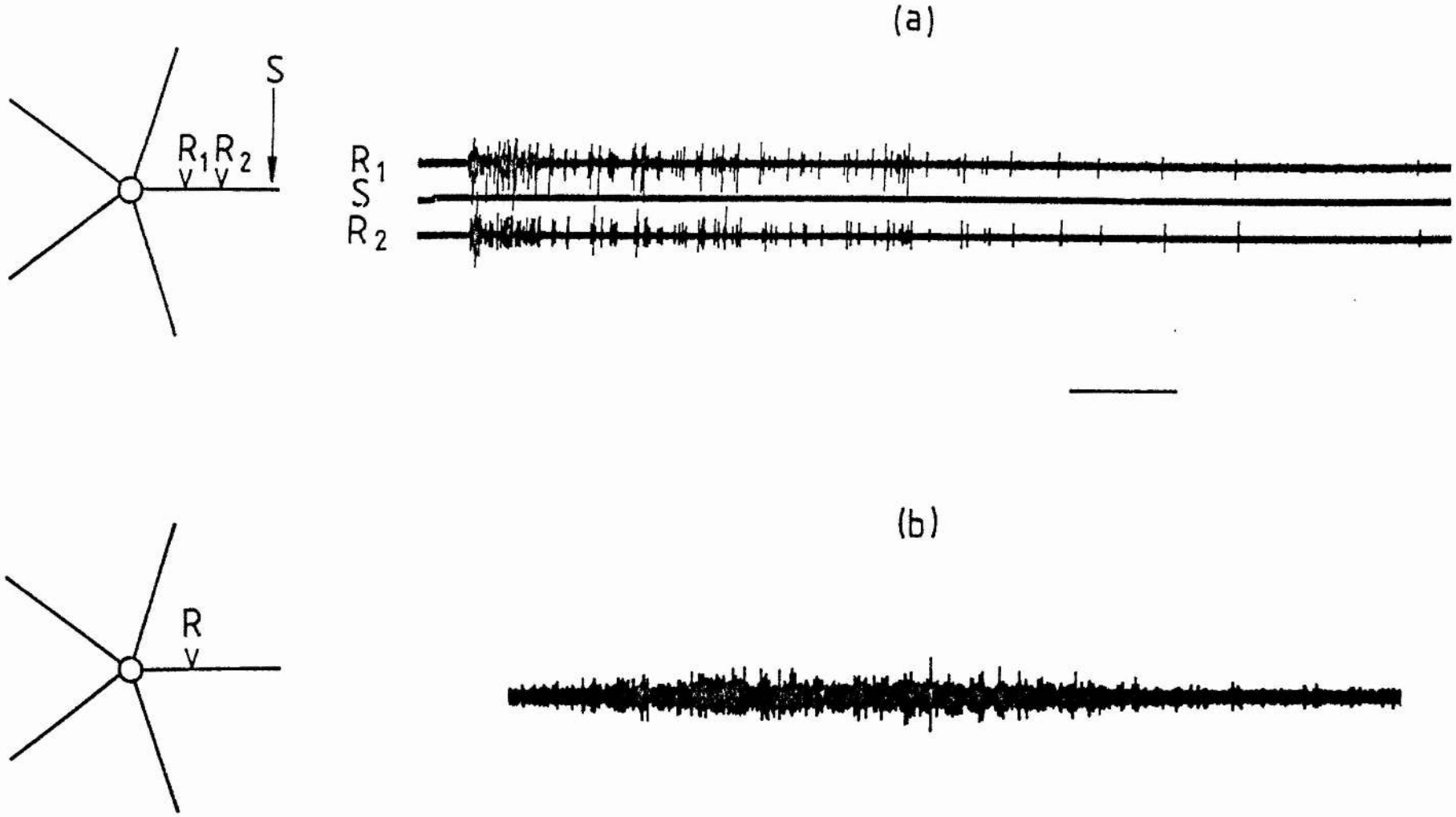

(a)
S
R1 R2
R1
S
R2
(b)
R

Fig. 16 shows the similarity between the responses thus obtained. Further experiments using inverted and non-inverted animals indicated that the oral and aboral surfaces of O.texturata had a similar sensitivity to light stimuli.

Responses to Chemical, Electrical and Mechanical Stimuli

The results of experiments in which the nervous system was stimulated electrically were variable; on occasions responses were obtained, but often electrical stimuli failed to elicit a recordable response. When successful, stimulation of the radial nerve cord with single electrical pulses of between 50 and 200 msec duration, and with an amplitude of 0.5-10 V evoked a response. Two types of response were observed. On occasion an electrical stimulus resulted in the repetitive discharge of a single unit (Fig. 17a), and in other instances electrical stimuli resulted in a massive discharge of a large number of units (Fig. 17b). In preparations from which electrical stimuli did evoke a response, increases in the amplitude and duration of the stimulus did not produce an alteration in either spike frequency or response duration. The amplitude and duration of the stimulus required to evoke a response varied between preparations. This variation may have been due to differences in current leakage, from the electrode to the surrounding seawater, in different preparations. Individual spikes were of a similar amplitude and duration to those elicited in response to photic stimulation, and the conduction velocity of

Fig. 16

Responses to light "OFF" were recorded from a single animal in both inverted and non-inverted positions, using the apparatus designed for an investigation of the righting reaction.
a) Response to light "OFF" with the animal inverted.
b) Response to light "OFF" in a non-inverted animal.

Time calibration = 1 sec

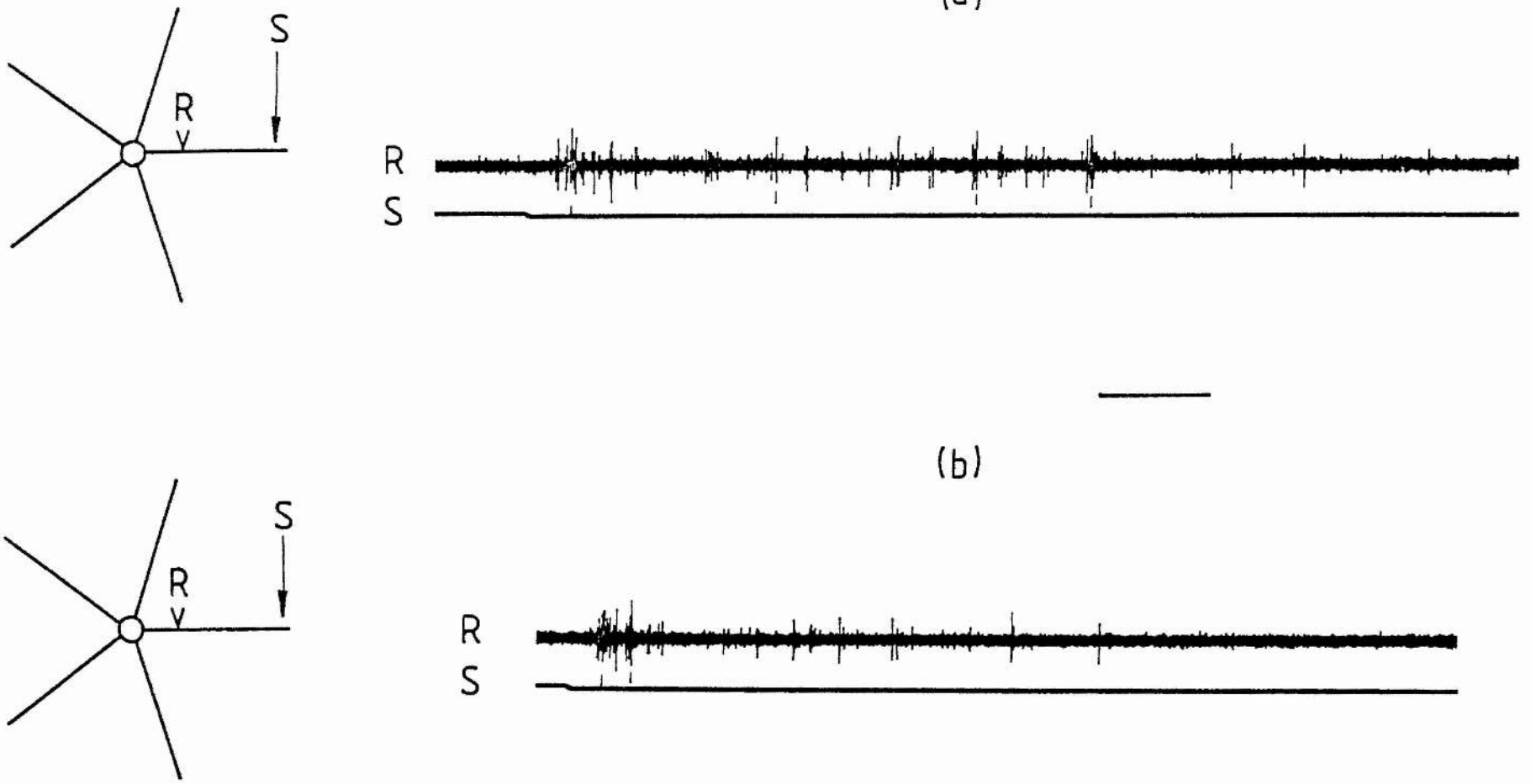
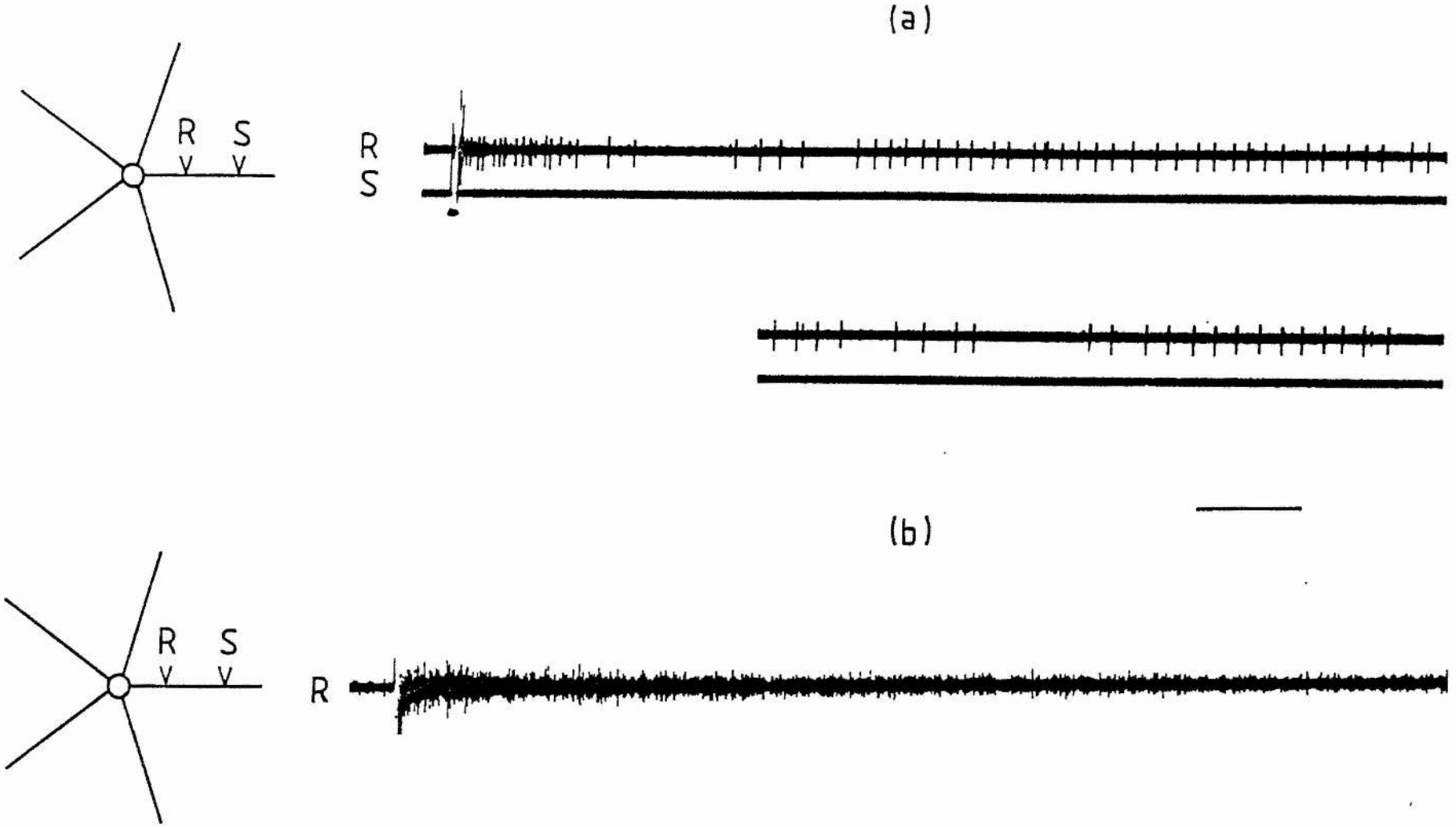

(a)
S
R
R
S
(b)
S
R
R
S

Fig. 17

Response to electrical stimulation of the radial nerve cords of O.texturata. Electrical stimulation did not always result in a detectable response within the nerve cords. When responses were elicited they were of one of two types.
a) Stimulation with a single pulse of 1.0 V and 200 msec duration, resulting in the repetitive discharge of a single unit.
b) Stimulation with a single 4.5 V pulse of 100 msec duration resulting in the discharge of a large number of units.

Time calibration = 1 sec


(a)
R
S
R
S
R
S
(b)
R
S
R

these spikes fell within the range previously determined for activity elicited in response to photic stimulation. Responses to repetitive electrical stimuli were not investigated.

Chemical stimuli, such a suspensions fish muscle tissue in seawater, applied to the preparation bath elicited a marked response (Fig. 18). Such responses attenuated only slowly with time, lasting for up to twenty minutes. A response could be re-evoked after flushing the preparation with fresh seawater for several minutes. The choice of fish muscle tissue as a chemical stimulus was based on behavioural observation; pieces of fish muscle placed in seawater tanks containing specimens of O.texturata, induce immediate feeding behaviour. The animals move rapidly in the direction of the food and on contact the food is immediately ingested. It was thus assumed that a suspension of fish muscle tissue in seawater would act as an effective chemical stimulus. The isolation of the component of the muscle suspension responsible for inducing a response was outside the scope of the present study.

Activity could also be evoked by mechanical stimulation of the experimental animal. Mechanical stimulation of an arm or rotation of the animal in a simulation of the "righting" response evoked a burst of single unit activity within the nervous system (Fig. 19). Such was the sensitivity of the experimental animals to mechanical stimulation that it was not possible to ascertain whether the response elicited by rotation of the animal, was due to inversion, or to the vibration inherent in moving the apparatus.

Fig. 18

Chemical stimulation. The application of a suspension of fish muscle tissue in seawater to the preparation resulted in a marked response which attenuated only slowly with time. The arrow indicates the point of application of the stimulus.

Time calibration = 1 sec

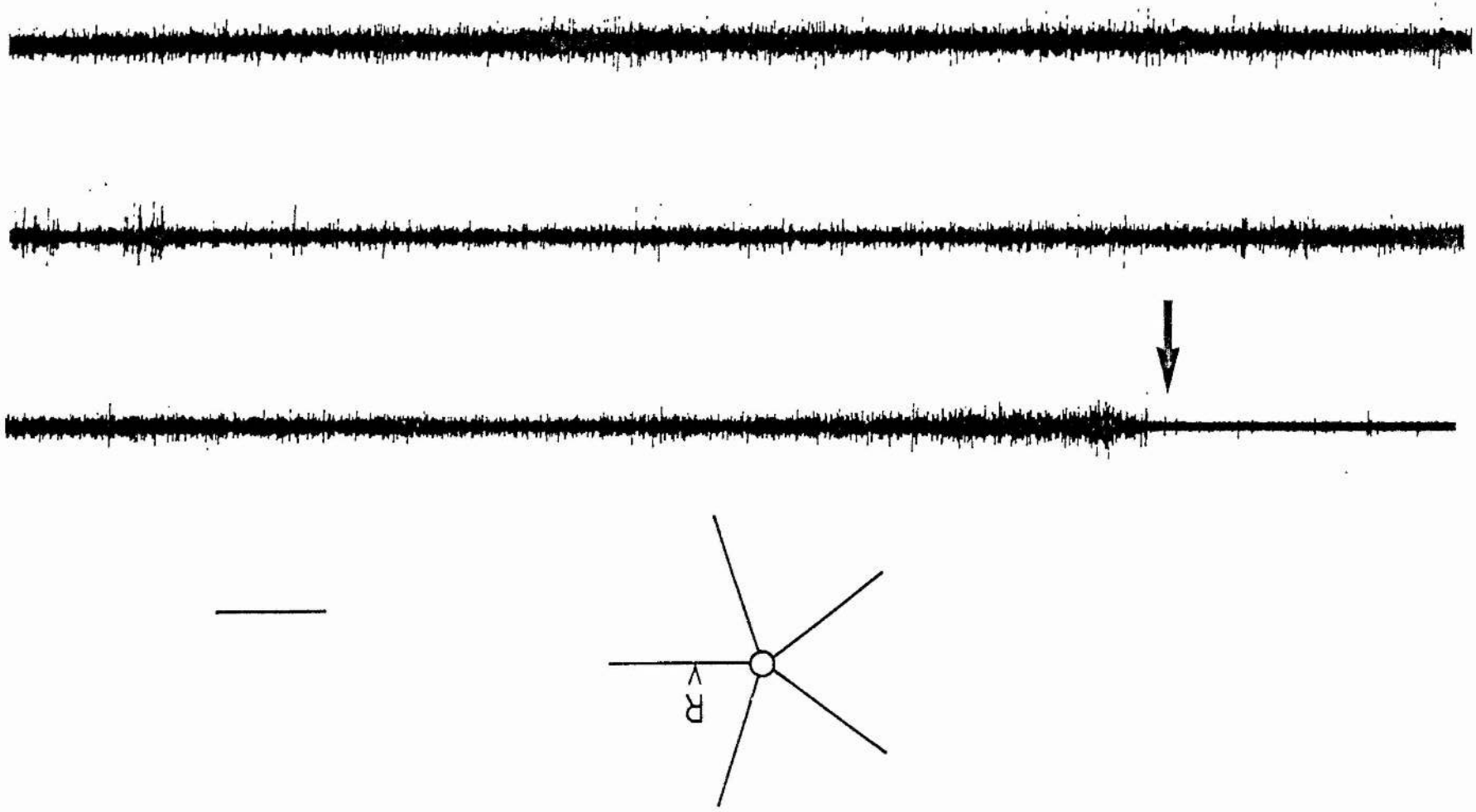
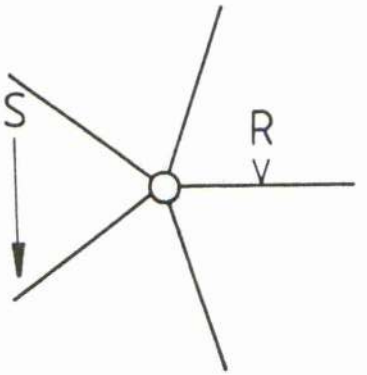

R

Fig. 19

Mechanical stimuli resulted in activity within the nerve cords. In this case the stimulus consisted of touching the tip of an arm with a glass rod. The arrows indicate the point at which the stimulus was applied.

Time calibration = 1 sec


S
R


R

Attempts to obtain extracellular recordings whilst simulating a righting response were therefore abandoned. The "righting apparatus" however proved useful in the comparison of photic responses from inverted and from non-inverted animals. Responses to chemical, mechanical and photic stimuli could not be recorded extracellularly from isolated radial nerve cords; electrical stimulation however did result, on occasion, in recordable activity within the nerve cord. A preliminary investigation of the ionic basis of the action potentials recorded from O.texturata revealed that all recordable activity was blocked by the replacement of normal seawater with calcium-free seawater (CaFSW). Electrical activity was restored by the addition of 10 mM calcium or by the replacement of the CaFSW with normal seawater. The blocking of transmission by the removal of calcium may suggest the presence of a calcium dependent action potential, or may be due to synaptic failure. Electrical activity within the nervous system was unaffected by the replacement of normal seawater by sodium-free seawater (NaFSW). An alteration in the temperature of the preparation had an effect on the observed response; a reduction of the temperature of the preparation to 1 ^{o}C totally abolished activity associated with photic stimulation.

2) Intracellular

Intracellular recordings were obtained from both ectoneural and hyponeural cells in the radial nerve cords. On impalement these cells registered resting potentials of between -40 and -70 mV. Spontaneously active cells in both tissues showed action potentials with amplitudes varying between 30 and 60 mV (Fig. 20). Some cells in both ectoneural and hyponeural tissues displayed action potentials with a very marked hyperpolarizing undershoot (Fig. 20). Many impaled cells in both tissues were "silent", but some could driven by current injection (Fig. 21). Synaptic potentials were recorded from a number of ectoneural and hyponeural cells (Fig. 22). Intracellular records of responses to light "OFF" stimuli were obtained from ectoneural cells in intact arm preparations (dissection method 3), on a number of occasions (Figs. 22 and 23).

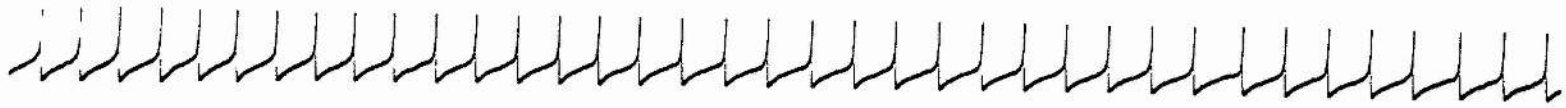
A number of problems were encountered during attempts to obtain stable intracellular recordings. Early attempts were made without the benefit of the use of the sliding plate manipulator and hydraulic advance system, and without electrodes pulled using a reliable electrode puller. Few penetrations were made under these circumstances and at best only injury discharges were recorded. Impalements were obtained more consistently when the sliding plate manipulator and hydraulic advance unit were used in combination with electrodes pulled using an Industrial Science Associates puller. Electrodes with tip resistances below 80 MΩ (thick-walled

Fig. 20

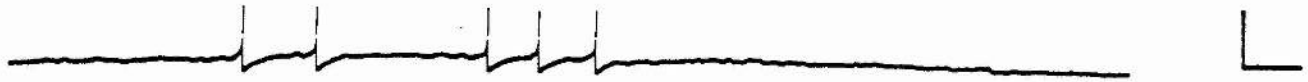
Intracellular recordings were obtained from both ectoneural and hyponeural neurones.
a) A record from a spontaneously active ectoneural cell.
Some ectoneural (b) and hyponeural cells (c) displayed action potentials with a marked hyperpolarizing undershoot.

Calibrations: vertical a,b,c = 60 mV
horizontal a,b = 100 msec; c = 200 msec

a)



b)



c)

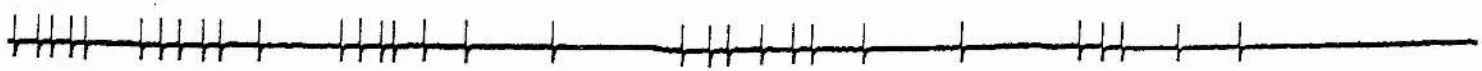


Fig. 21

Many of the impaled cells were silent, but some could be driven by current injection.
a) Constant depolarizing current injection of 0.5 nA caused this ectoneural cell to fire.
b) In this instance constant depolarizing current injection (0.5 nA) produced spikes in an ectoneural cell. The small amplitude of these spikes suggests that they may be an electrotonically conducted reflection of spikes elsewhere in the cell.
c) The response of a hyponeural cell to constant depolarizing current injection of 1.0 nA.

Calibrations: vertical a,c = 40 mV; b = 20 mV
horizontal a,b,c = 200 msec

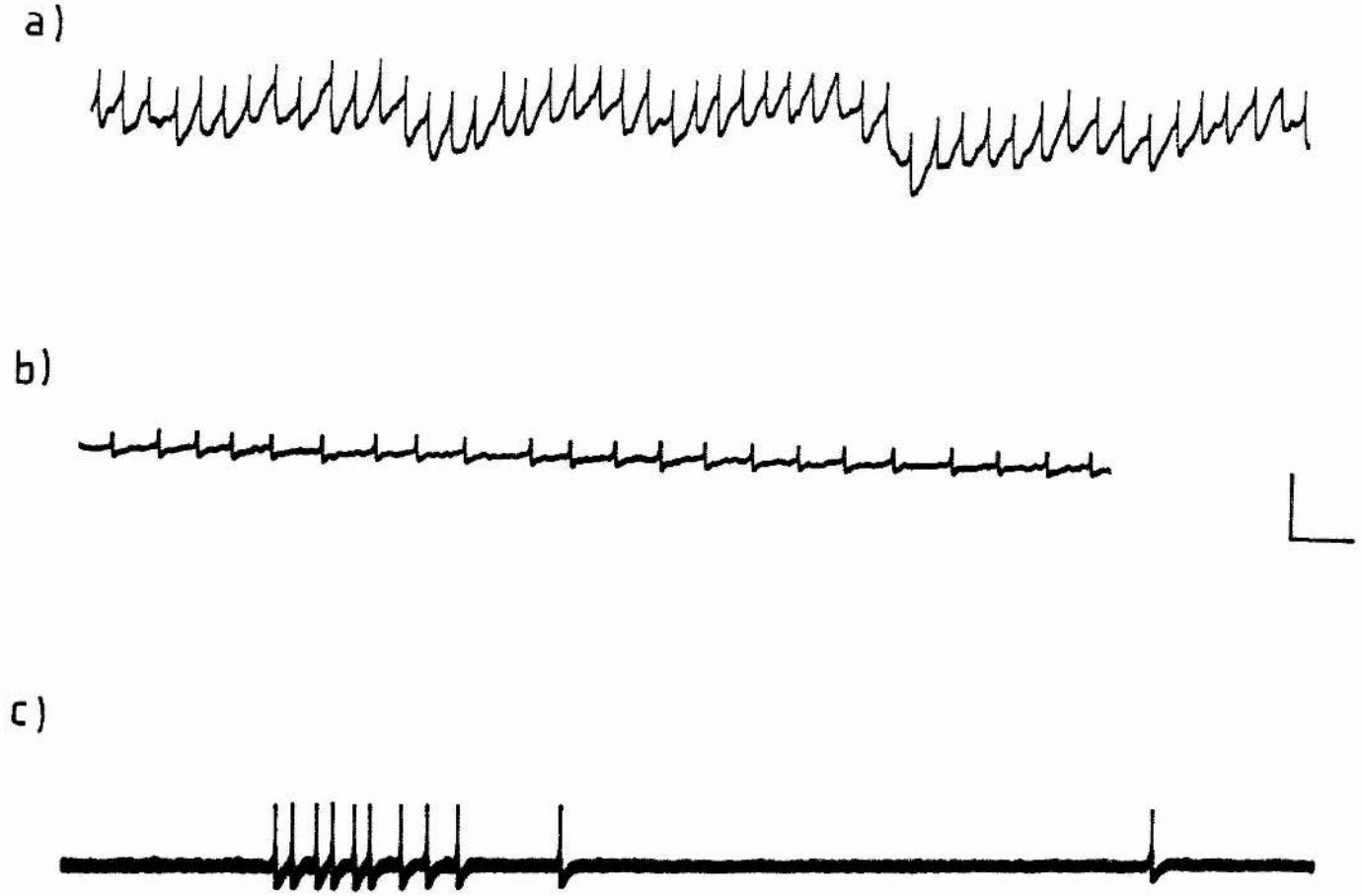

a)
b)
c)

Fig. 22

A number of cells in both the ectoneural and hyponeural tissues showed evidence synaptic activity.

a) epsp´s in an ectoneural cell.

b) epsp´s recorded from a hyponeural cell in response to a light "OFF" stimulus.

Calibrations: vertical a,b = 5 mV
horizontal a,b = 200 msec

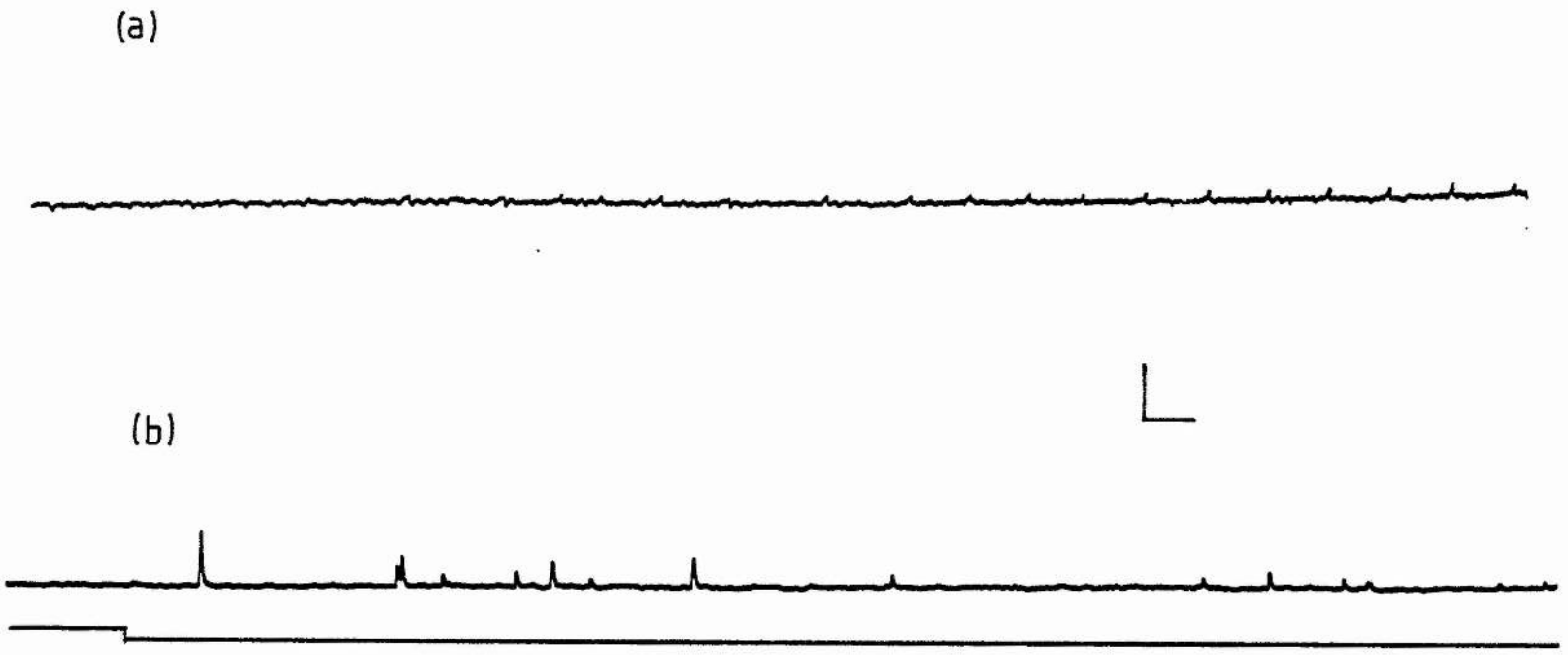
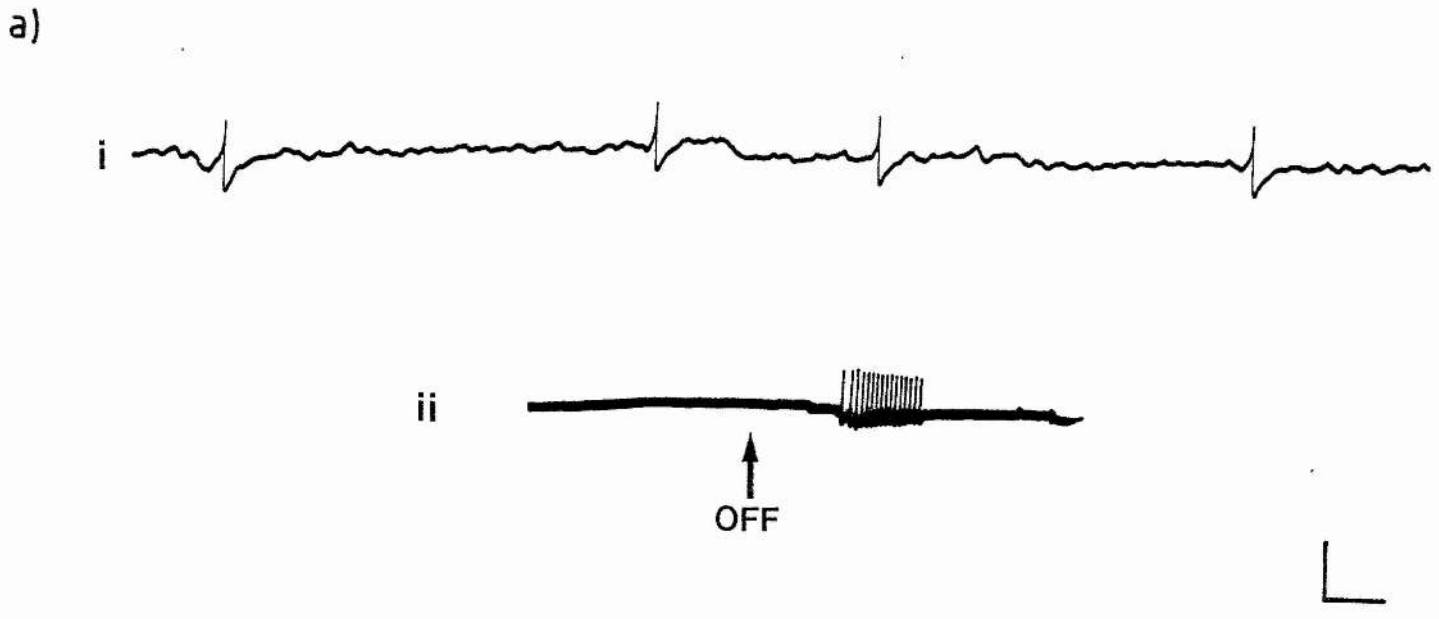

(a)
(b)

Fig. 23

On a number of occasions intracellular records of a response to a light "OFF" stimulus were obtained.
a) Activity to a light "OFF" stimulus in two ectoneural cells
b) epsp´s recorded from an ectoneural cell in response to light "OFF".

Calibrations: vertical 40 mV
horizontal a = 100 msec; b = 200 msec


a)
i
ii
OFF
b)

glass, 2 M KCl) were found to be ineffective.

Two main problems were encountered: The characteristics of the electrode changed markedly subsequent to the initial penetration, and further impalements using the same electrode were rare. There was no measurable increase or decrease in the electrode resistance after the initial penetration, so it did not appear that the electrodes were blocked or that the tip had been damaged. Inspection of the electrodes with the light microscope revealed no gross damage to the electrode. Attempts to examine electrodes with the scanning electron microscope were not successful, due to a tendency of electrons to stream from the tip of the electrode, thus obscuring the details of the shape of the electrode tip. The possibility that the electrodes were being partially blocked by coagulation of protein around the electrode tip due to current leakage was considered. This explanation was discounted because of the absence of leakage current from the D.C.amplifier. Attempts to penetrate cells whilst passing small (0.25 - 1 nA) amounts of either hyperpolarizing or depolarizing current, to offset any undetected current leakage, were no more successful than before. The possibility that the electrodes were being partially blocked by mucus on the exterior surface of the preparation was countered by repeatedly flushing the preparation with fresh seawater and, on occasion, by a variety of enzyme treatments (application of pronase, trypsin or N-acetyl L-cystiene). Neither flushing the preparation with fresh seawater nor treatment with enzymes improved the performance of the electrode.

The second major problem encountered was an inability to "hold" penetrated cells for periods of greater than four or five minutes. This problem was not caused by electrode drift as the manipulator and advance system were capable of holding an electrode in position without movement for at least 12 hours.

The inability to obtain stable penetrations hampered attempts to produce dye fills of impaled cells. Attempts to fill cells with lucifer yellow were unsuccessful, although on a number of occasions dye was apparently passed into cells for up to five minutes without electrode blockage. Small nerve cells in planarian brains have been filled with lucifer in as little as two minutes (Keenan et al., 1981), and the reasons for the failure of attempts to fill cells in O.texturata is not known. Attempts to fill cells with cobalt or nickel chloride were also not successful; in all cases electrode blockage occurred within one or two minutes. Injection of either hyperpolarizing or depolarizing current pulses of greater than 1 or 2 nA often resulted in the loss of the impalement.

Intracellular recordings were obtained from all three types of preparation that were described in the materials and methods section of this chapter. The most consistent results were obtained when isolated radial nerve cords were used (dissection method 1); in these instances recordings were made from ectoneural cells. Problems associated with dissecting the nerve cord free from the arm without incurring serious damage to the tissue were overcome by staining the nerve cord with the vital stain, neutral red.

Successful impalements were obtained using beveled electrodes, however the beveling process did not appear to improve the performance of the electrodes and it was eventually abandoned because of the increased time required to produce beveled electrodes. The use of a piezoelectric pulsing device (Chen, 1978) to aid in the penetration of cells was eventually abandoned. The device was used in attempt to produce an axial advance of the microelectrode into a cell. It was hoped that this would prevent damage to the cells which may have been caused by the tip of the electrode moving in a lateral direction across the surface of a cell. However in spite of the designer's claims, alignment of the piezoelectric disc to produce an axial movement of the electrode proved impossible.

DISCUSSION

Decrementally conducted compound potentials were not recorded from the radial nerves of O.texturata (or from O.fragilis, or from O.nigra) even when the recording electrodes were separated by only one segment of the nerve cord. This contrasts with Brehm's (1977) report of decrementally conducted compound potentials, as well as single unit activity within the radial nerves of Ophiopsila californica. All the published reports to date of electrical activity within the echinoderm nervous system, with the exception of those of Brehm (1977) and of Takahashi (1964), have dealt solely with decrementally conducted activity (Sandeman, 1965; Millott and Okumura, 1968; Binyon and Hasler, 1970; Podol'skii, 1972). In these studies the recorded potentials had an amplitude that was graded with the stimulus intensity, and that showed a logarithmic loss of amplitude with distance. The physiological basis of these decremental potentials remains unclear. A description of the conduction of these compound potentials does not explain the way in which excitation spreads rapidly over long distances within the echinoderm nervous system.

Through conducted single unit activity has however been reported in two separate studies prior to this present account. In the first of these a burst of single unit spikes was recorded from the isolated radial nerve cord of the sea urchin, Diadema setosum, with the aid of glass-insulated silver microelectrodes, in response to photic stimulation (Takahashi, 1964). The considerable delay in the onset of a response after the application of the stimulus has

not been explained. The results of experiments using similar electrodes, in an attempt to repeat Takahashi's work suggest that the apparent unitary activity is the result of a photoelectric effect of the electrodes (Valentincic, personal communication). Brehm's work is thus the only bona fide report of single unit activity within the nervous system of an echinoderm. Spike-like potentials have been recorded from muscle tissue in echinoids (Cobb, 1968; Peters and Shelton, 1981) but a nervous origin for these impulses is unlikely since in both cases the muscle innervation was confined to areas well removed from the recording sites.

In contrast to the experiments with O.californica (Brehm, 1977), from which single unit activity was only recorded in response to electrical stimulation, spike activity could be recorded from O.texturata in response to electrical, chemical, mechanical and particularly to photic stimulation. Photic stimuli were used for the bulk of the extracellular investigations of conduction within the nervous system of O.texturata, because of the relative ease and reliability with which a response could be obtained.

It has long been known that echinoderms respond to light, all classes have received attention but the asteroids and echinoids have been most favoured in this respect. The intractability of echinoderms for electrophysiological study has meant that

photosensitivity has, to date, been largely inferred from the results of behavioural studies (von Uexkull, 1905; Cowles, 1910; Holmes,1912; Millott, 1954; Dimelow, 1958), Photic stimulation of an arm of O.texturata resulted in a burst of spikes that could be recorded from any point along the radial nerve cords and circumoral nerve ring. Experiments in which the circumoral nerve ring was cut to one side, or on both sides of the stimulated arm demonstrated that information was conducted in both directions around the circumoral ring. This confirms Kerkut's (1954) conclusion that circumoral conduction is bidirectional.

Extracellular recordings can be made from the radial nerve cords of ophiuroids, but similar recordings cannot be obtained from asteroids or echinoids. This, coupled with the fact that the Ophiuroidea is the only echinoderm class in which giant nerve fibres are found, suggests that single unit activity recorded from brittlestars is activity from within the ectoneural giant fibres. Further, the fact that the site of photoreception is located peripherally, and not within the nerve cords, suggests that the ectoneural giant fibres are a class of interneurones. This conclusion is supported by the absence of ectoneural giant fibres in any of the nerve branches from the main nerve cords. The apparent lack of an alteration in the pattern of the photic response as it travels along the nerve cords suggests that the ectoneural giant fibres carry similar information to each ganglion; it is not clear whether separate pathways transmit photically, chemically and mechanically elicited responses, or whether the system has a more general role in which information from a wide

variety of stimuli is transmitted via the same pathway. Integration of the response might occur at a number of levels; initially the response may be integrated in the radial nerve cord ganglion nearest the point of stimulation, resulting in the transmission of an integrated response to all other parts of the nervous system. This would explain the lack of alterations in the response pattern. Further integration could occur in each radial nerve cord ganglion, involving smaller ectoneural units from which extracellular recordings are not made, and at the level of the generation of the hyponeural motor output.

The anatomical studies reported in chapter 1 of this thesis suggest that the structure of the circumoral ring is consistent only with providing a functional connection between adjacent radii, and not with the task of central integration. The extracellular records obtained from the circumoral nerve ring lend support to this view. The response to photic simulation is conducted round the circumoral ring and there is little sign of an alteration in the pattern of the response during this process. This observation is apparently inconsistent with the model of circumoral conduction derived from studies of pharyngeal retractor muscle contraction in Cucumaria (Pople and Ewer, 1955). From this model one would expect record fewer units from the segments of nerve ring further away from the stimulated arm, than from those adjacent to the stimulated arm.

Brittlestars are known to be negatively phototactic (Cowles, 1910), and the finding that O.texturata is maximally sensitive to the wavelengths of light normally encountered in marine habitats, and at intensities within the range found in coastal waters, correlates well with this phototactic behaviour. The greater sensitivity to light of the arm tips as compared with the arm bases, may be linked in some way with the behavioural observation that O.texturata often buries itself in sand or mud, leaving only the tips of the arms projecting into the water column. Since more than one segment of the arm must be photically excited before a response can be detected it would seem that input from the photoreceptive system of more than one arm segment, to the radial nerves, is required to trigger the propagation of a light response through the nervous system. The inability to record a photic response from isolated radial nerve cords or from preparations from which the dermal plates had been removed suggests that the nervous elements themselves are not photosensitive, and that the photoreceptive sites are located peripherally, probably embedded in the dermal plates. The conclusion that that the radial nerve cords are insensitive to light is apparently at odds with the results obtained by Millott and Yoshida (1959), who found that the radial nerves of Diadema antillarum could be stimulated by light, resulting in vigorous movements of the spines. It is possible that during these experiments the sensory cells in the epithelium were also being stimulated by the light beam used to excite the nerve cord. Ultrastructural studies of the light sensitive areas of the skin of Diadema antillarum have revealed no structure resembling a

clearly defined photoreceptor (Millott and Coleman, 1969). The only clearly defined photoreceptors reported in echinoderms are the optic cushions of starfish, and they are the most complex sense organs so far described in echinoderms. The photosensitivity of these organs has been inferred from their structure (Vaupel von Harnack, 1963; Eakin,1963; Penn and Alexander, 1980) and to some extent from behavioural observation (Hartline et al., 1952), direct electrophysiological evidence however has not been obtained.

The absence of clearly defined photoreceptors from the body wall of ophiuroids suggests that, as in echinoids, photosensitivity in ophiuroids is mediated by a form of dermal light sense. The dermal light sense in echinoids has been attributed to the numerous nerve fibres that penetrate the epidermis (Millott, 1975) and it is probable that light detection by ophiuroids involves sensory neurones embedded in the dermal plates.

The responses to photic stimulation recorded from animals restrained in the more natural "aboral side uppermost" position were apparently similar to those recorded from animals restrained with the oral surface uppermost. Since the nervous system in brittlestars lies close to the oral surface of the animal and is thus more accessible with the animal held in an inverted position the majority of the experiments reported in this study were performed on inverted animals. The fact that responses to photic stimulation could be recorded from species of brittlestar other than O.texturata indicates that that the light response is widespread in brittlestars, and that it is not merely a

physiological quirk occurring only in a single species.

Responses elicited by chemical, photic and mechanical stimuli demonstrate that even though clearly defined sense organs have not been described in ophiuroids, they are capable of detecting a wide range of sensory stimuli. Further work is required to determine the range and levels of stimulation required to elicit a response. The inability to record responses reliably to electrical stimulation of the nerve cords in O.texturata is puzzling. The absence of recordable single unit activity in response to electrical stimulation in other echinoderms has been ascribed to the extremely high extracellular resistances, caused by the tight packing of the nerve fibres and by the absence of glia or blood spaces (Pentreath and Cobb, 1972). Responses to electrical stimuli in O.californica were apparently reliably recorded by Brehm (1977) and it should be possible to stimulate the giant cells in O.texturata electrically. The fact that on occasions a response could be elicited points to an inadequacy in the experimental technique. The problem probably lies in the use of suction electrodes for stimulation, since this type of electrode is not isolated from the bathing medium and considerable current leakage can occur.

The conduction velocity of extracellularly recorded unitary activity O.texturata is similar to the value of 780 mm/s for the conduction velocity of single unit activity within the radial nerves of O.californica (Brehm, 1977). Conduction velocity values for decrementally conducted potentials in asteroids and echinoids range between 60 and 300 mm/s and this, combined with the long duration and large amplitude of decrementally conducted potentials clearly distinguishes them from single unit activity. The abolition of recordable activity by the removal of calcium from the external medium may indicate the presence of a calcium dependent action potential. Brehm (1977) reported the presence of a calcium dependent action potential in O.californica, and both Millott and Okumura (1968) and Binyon and Hasler (1970) suggested that compound activity in starfish and sea urchins may be calcium dependent. However the removal of calcium from the external medium is known to cause the failure of synaptic transmission (Katz and Miledi, 1967a), and the abolition of recordable activity in O.texturata may well have been due to the disruption of synaptic transmission, rather than to the absence of the ionic requirements of the action potential. Removal of sodium from the extracellular medium apparently has no effect on transmission within the nervous system of O.texturata. Spike activity in O.californica was also unaffected by the removal of extracellular sodium. The blocking of activity by removal of calcium, coupled with the fact that removal of sodium does not block conduction, implies the presence of a calcium spike. The blocking of recordable activity by the lowering of the temperature of the preparation to 1 ^{o}C was probably due to

synaptic failure. Low temperature is known to cause the failure of synaptic transmission at the frog neuromuscular junction (Katz and Miledi,1965b).

The fact that single units can be recorded from a number of widely spaced points along the nerve cords indicates, from a functional point of view, that ophiuroid nerve cords contain long single units. The ultrastructural studies described in chapter 1 show that the radial nerve cords contain only very short fibres, and it is therefore necessary to conclude that the long functional units consist of a large number of synaptically coupled neurones. It is not clear whether these long functional units rely on chemical synapses or on electrotonic coupling of the cells. The presence of electrotonic junctions was not detected during the work described in the ultrastructural part of this study, nor have they been previously described in any adult echinoderm. Some delay in the rate of conduction would be introduced by the large number of chemical synapses required to form the comparatively short ectoneural neurones into long functional units. However the delay thus introduced would not be inconsistent with conduction velocity determined for single unit activity in O.texturata.

The use of suction electrodes for recording purposes proved to be the only way to record extracellularly from the ophiuroid nervous system. Some problems were associated with their use. The lack of mechanical strength of the nerve cords meant that some damage resulted when the suction electrodes were detached from the nerve cord subsequent to recording. This in turn meant that

separate preparations had to be used for each experiment. The electrodes were found to work best when the tip diameter was just smaller than the width of the nerve cord. Records obtained were thus from the whole nerve cord at a particular point, and the activity from a large number of units was inevitably recorded. Attempts to localize responses using suction electrodes with smaller diameter tips were unsuccessful, because of damage to the nerve cord. Variations in the precise manner in which the elctrode was attached to the nerve cord were probably responsible for the differences in the responses recorded from different preparations in response to identical stimuli. Results from different preparations were thus not directly comparable.

Intracellular recordings from echinoderm neurones have not previously been reported. All the reported electrophysiological studies of echinoderm nervous systems, apart from Brehm's (1977), have utilized either echinoids or asteroids. The small size of the neurones which make up the nerve cords of these animals effectively precludes their use for intracellular studies. The size of the giant neurones in ophiuroids offers, from a theoretical point of view at least, the opportunity of carrying out intracellular studies on echinoderm nerve cells.

The intracellular studies reported in this chapter amount to little more than a feasibility study; the results demonstrate that it is possible to make intracellular recordings from single ophiuroid neurones, although for the present two problems remain to be overcome. The nature of the damage sustained by the electrode during an initial impalement is not yet clear. The absence of an increase or decrease in the measured resistance of the electrode after the initial impalement, suggests that the tip of the electrode is neither blocking nor breaking off. The damage may of course be a combination of breakage and blockage resulting in little alteration of the electrode resistance. Constraints of time and finance prevented the construction of a device that would have allowed non-destructive examination of the electrodes with a scanning electron microscope. Modifications to the stage of a Cambridge scanning electron microscope have allowed electrodes to be successfully examined without incurring damage (Fry, 1975; Baldwin, 1980). Examination of the electrodes before and after an experiment should yield some clue to the nature of the damage sustained by the electrode during an initial impalement. The problem of sustaining an impalement for more than four or five minutes is also likely to be linked to the performance of the electrode. It seems probable that the inability to "hold" cells for longer than a few minutes is the result of damage sustained by the cell on impalement, resulting ultimately in a decay of the resting potential.

In spite of the problems encountered during the intracellular work described in this study, successful impalements, lasting several minutes, could be obtained on a regular basis. With further minor modifications to the experimental technique, including particularly an improvement in the performance of the electrodes stable impalements should become routine.

Intracellular studies are required to answer a range of outstanding questions about the structure and function of the echinoderm nervous system. It should be possible to determine the ionic basis of the action potential, and to investigate the role of the giant neurones in the conduction of a variety of responses in ophiuroids. In particular, dye-filling experiments should elucidate the three dimensional shape and connections of individual giant cells, a task which is not practicable using microscopical techniques alone (see chapter 1 - Discussion).

The groups of ectoneural and hyponeural cell bodies which stain intensely with neutral red deserve further investigation. It is possible that they contain catecholamines or 5-hydroxytryptamine, as do the cells in leech ganglia which are similarly stained by neutral red (Stuart et al., 1974) Catecholamines have been detected in the ectoneural tissue of the radial nerve cords of Ophiothrix fragilis, but not in the hyponeural tissue (Pentreath and Cottrell, 1970). In this latter study the fluorescent ectoneural cell bodies were found to centred around the mid-line of the nerve cord. Catecholamines have also

been detected in the nerve plexus underlying the epidermis covering the genital shields in O.texturata (see chapter 3). 5-hydroxytryptamine has been detected in small quantities in asteroids, echinoids and holothuroids (Welsh and Moorhead, 1960; Welsh, 1966), but not in ophiuroids. Fluorescence histochemical studies, particularly using the sensitive glyoxylic acid (de la Torre and Surgeon, 1976) and aluminium-catalysed formaldehyde (ALFA) techniques (Ajelis et al., 1979) should shed further light on this problem.

CHAPTER 3

A PREVIOUSLY UNKNOWN CILIARY FEEDING STRUCTURE IN *Ophiura texturata*.

INTRODUCTION

Ophiuroids exhibit a variety of feeding methods which encompass direct absorption via the body wall, feeding on both small and large particulate matter, as well as the capture of small prey organisms (for a review see Reese,1966). Most of the feeding methods involve the use of the arms and podia, or water currents produced by the cilia of the test epithelium and the gut endothelium. The most detailed information on the feeding habits and methods of ophiuroids comes from extensive work with *Ophiocomina nigra* (Mortensen, 1927; Smith, 1937; Vevers, 1956 and Fontaine, 1964 and 1965). *O.nigra* is omnivorous and feeds on algae, fish, foraminfera, annelids and molluscs as well as on carrion. In areas where strong water currents occur the arms are raised into the water column and suspended food particles are trapped by mucus secreted by the podia. The mucus often becomes thick enough to form a net between arm spines. The podia gather the food laden mucus and direct it toward the mouth. This is known as mucus-net feeding (Fontaine, 1965). *Ophiothrix fragilis* also shows a diversity of feeding methods and carbon-14 labelled phytoplankton has been used to demonstrate filter feeding in this animal (Rhoushdy and Hanson, 1960), and filter feeding in the pacific brittlestar *Ophiopholis aculeata* has been described by

LaBarbera (1978). Ophiura texturata has been documented as feeding mostly on detritus and carrion (Blevgad, 1914), and recent work by Feder (1981) has confirmed Blevgad's report. An early published description of feeding by O.texturata (Wintzell, 1918) included a description of a feeding method in which food particles were swept by ciliary action into the mouth. This latter report did not however contain an accurate, detailed description of the structures responsible for producing the ciliary current. Suspension feeding behaviour, similar to that shown by other species of brittlestar (Roushdy and Hanson, 1960; Fontaine, 1965) has been suggested for O.texturata by Rasmussen (1973).

This chapter describes a ciliated structure possessed by O.texturata, which appears to be specialized for a form of ciliary mucus feeding; the bursal slits in O.texturata are covered by a precisely orientated arrangement of ciliated ridges and non-ciliated grooves. Numerous mucous cells are associated with this structure, and water currents created by the cilia move food particles toward the mouth, and thence to the stomach. The calcite hooks that were reported to be present on the stomach wall in O.texturata (Feder and Larsson, 1968) were not observed during this study.

The significance of the innervation of this structure is discussed with particular reference to its' importance as a preparation for the study of the control of ciliary beating and mucus secretion in echinoderms.

MATERIALS AND METHODS

Light and Transmission Electron Microscopy

Tissue for light and transmission electron microscopy was fixed using two techniques. The first method involved a single fixation stage using 1% phosphate-buffered osmium tetroxide solution for 1 hour (for detailed method see Chapter 1). The second technique involved pre-fixation in an osmium tetroxide/gluteraldehyde mixture. Fixation was carried out in 4% gluteraldehyde in 0.2M sodium cacodylate with 0.1M sodium chloride and 0.35M sucrose added. 1% osmium tetroxide in 0.2M sodium cacodylate with 0.3M sodium chloride was used for post-fixation (Eisenman and Alfert, 1982). The fixed tissue was rinsed in 0.36M sodium chloride and was subsequently decalcified in a 1:1 mixture of 2 % ascorbic acid and 0.36M sodium chloride for 24 to 48 hours (Dietrich and Fontaine, 1975). The decalcified material was washed in distilled water, dehydrated in acetone and finally embedded in Durcupan. Light microscopic sections were stained with toluidene blue prior to examination. Ultrathin sections were stained with uranyl acetate and lead citrate and were examined with an AEI EM6B operated at 60 KV.

Scanning Electron Microscopy

Tissue for scanning electron microscopy was fixed in 1% osmium tetroxide solution in seawater for 15 minutes and was then washed in running tap water for 5 minutes to remove any mucus. The fixed material was dehydrated in alcohol, prior to being critically point dried. The specimen was then sputter coated with gold and examined using a Cambridge S-600 stereoscan.

Fluorescence Histochemistry

A modification of the Falck-Hillarp technique (Falck and Owerman, 1965) was used to demonstrate the presence of biogenic amines. Pieces of genital shield were frozen in liquid propane maintained at a temperature of -196 ^{o}C by immersion in liquid nitrogen and were then placed in a freeze dryer at -40 ^{o}C and at a vacuum of 10^{-2} Torr, for five days. The tissue was subsequently exposed to formaldehyde (70% relative humidity) for one hour at 80 ^{o}C, prior to being embedded in Spurr's resin (Spurr, 1966); whilst in the embedding resin, and prior to polymerization, the epithelium covering the genital shields was dissected free from the underlying calcite, which was then discarded. Thick (1-2 μm) sections were mounted in immersion oil and examined with a Zeiss transmission fluorescence microscope, using Zeiss BG 12 and 44 and 52 filters.

RESULTS

Observations on living animals

The bursal slits in Ophiura texturata extend on either side of each arm from the periphery of the disc to the buccal shields. The sides of the bursal slits are formed by the genital shields and the slits open into an invaginated pouch of the body wall, the bursa, which is thought to have a genito-respiratory function (see Hyman, 1955 for a review).

Examination of parts of living specimens of O.texturata with the light microscope revealed that powerful ciliary currents are produced across the genital shields. Examination of sections of fixed material with the light microscope show that the genital shields are covered by horizontally arranged rows of cilia above a highly structured epithelium.

Experiments carried out with living specimens of O.texturata, using suspensions of carmine particles in seawater showed that the beating of the cilia produces a strong inward current from the aboral disc surface into the bursal slits, and an outward current through the slit between the arm and the disc, which is directed orally. The bursal cavity itself is lined by ciliated epithelium and circulating currents occur within the bursa. The main current produced by the ciliated ridges on the genital shields however does

not enter the bursa, but is directed towards the mouth. Substantial amounts of mucus are secreted by the cells of genital shields and food particles trapped in the mucus are transferred to the mouth from the bursal slit by the ciliary currents and the podia. The podia adjacent to the bursal slit have a stereotyped movement. They flex distally in such a way that they are able to direct the food laden mucus towards the mouth.

Pharmacology

Preliminary pharmacological experimentation has suggested that ciliary beating is not under nervous control but that the production of mucus may be. The application of various transmitter substances, noradrenalin, dopamine, acetylcholine and 5-hydroxytryptamine in concentrations between 10^{-3} M and 10^{-5} M, to genital shield preparations did not produce an alteration in the frequency of ciliary beating. However, concentrations of dopamine and noradrenalin as low as 10^{-7} M were found to effect the secretion of mucus; noradrenalin produced a reduction in the amount of mucus secreted and dopamine resulted in a marked increase.

Structure of the Epidermis

The epidermis of echinoderms consists of a fibrous or sometimes granular cuticle overlying a layer of epithelial cells. The structure of echinoderm cuticle has been previously described (Hyman, 1955), and Holland and Nealson (1978) have recently provided a detailed description of the cuticle in all five echinoderm classes, and have reviewed the previous literature. They showed that the cuticle of ophiuroids is different from that of asteroids, crinoids and echinoids. In ophiuroids few microvilli penetrate the cuticle but large numbers of detached microvillar caps are embedded in it. The non-cellular connective tissue below the cuticle and above the epithelium is much more extensive in the ophiuroids than in the other three classes. Holland and Nealson (1978) discussed the application of various fixation techniques for the preservation of echinoderm cuticle. They pointed out that the use of gluteraldehyde might destroy the cuticle. In the present study the fixation technique using a gluteraldehyde/osmium mixture prior to the main fixation stage resulted in improved preservation of the tissue; the vacuolation of the epithelial and secretory cells produced by osmium fixation was much reduced and the preservation of the cuticle was improved. The gluteraldehyde/osmium mixture however produced poor fixation of nervous tissue and of the cytoplasmic detail of the ciliated cells. It would therefore seem to be necessary to employ a variety of fixation techniques when the ultrastructure of echinoderm tissue is to be investigated,

especially where nervous tissue is involved.

In the region of the bursal slits the epidermis of O.texturata more closely resembles that previously described in echinoids and asteroids (Kawaguti and Kamashima,1964; Cobb,1968; de Sousa Santos and Sasso,1970; Holland and Nealson,1978) (Fig. 4). The microvilli are numerous and are always associated with microvillar caps. There is little connective tissue between the cuticle and the epithelial cells. There are large numbers of different secretory cells between the epithelial cells and the distribution of these has been extensively described; De Sousa Santos in a series of papers,1966a&b, 1968 and 1970, has described gland cells in echinoderms and has named these three types A,B and C cells. Martinez has more recently described up to six types of gland cells in ophiuroids and named them A-F (Martinez,1976 and 1977).

The epithelial tissue is further specialized over much of the genital shields into a series of ridges and grooves. The ridges which run horizontally are covered by a broad band of motile cilia. Each ridge measures approximately 20μm in width and is covered by 18 to 20 rows of cilia which run the entire length of the ridge. The grooves between the ridges are approximately 120 μm wide. The grooves and the cilia are illustrated by scanning micrographs Figs. 1 and 2. Figure 3 illustrates part of a ciliated ridge with non-ciliated secretory epithelial cells at the edge of the ridge. The secretory and epithelial cells contain large electron transparent areas, which are often present in fixed

Fig. 1

Individual cilia about 20 deep cover the ridges which extend in parallel tracts across the genital shields of both the arm and the disc.

x 750

Fig. 2

Scanning micrograph of the ciliated tracts lining the buccal shields in Ophiura texturata to show the ridged appearance of the surface of the genital shields.

x 100

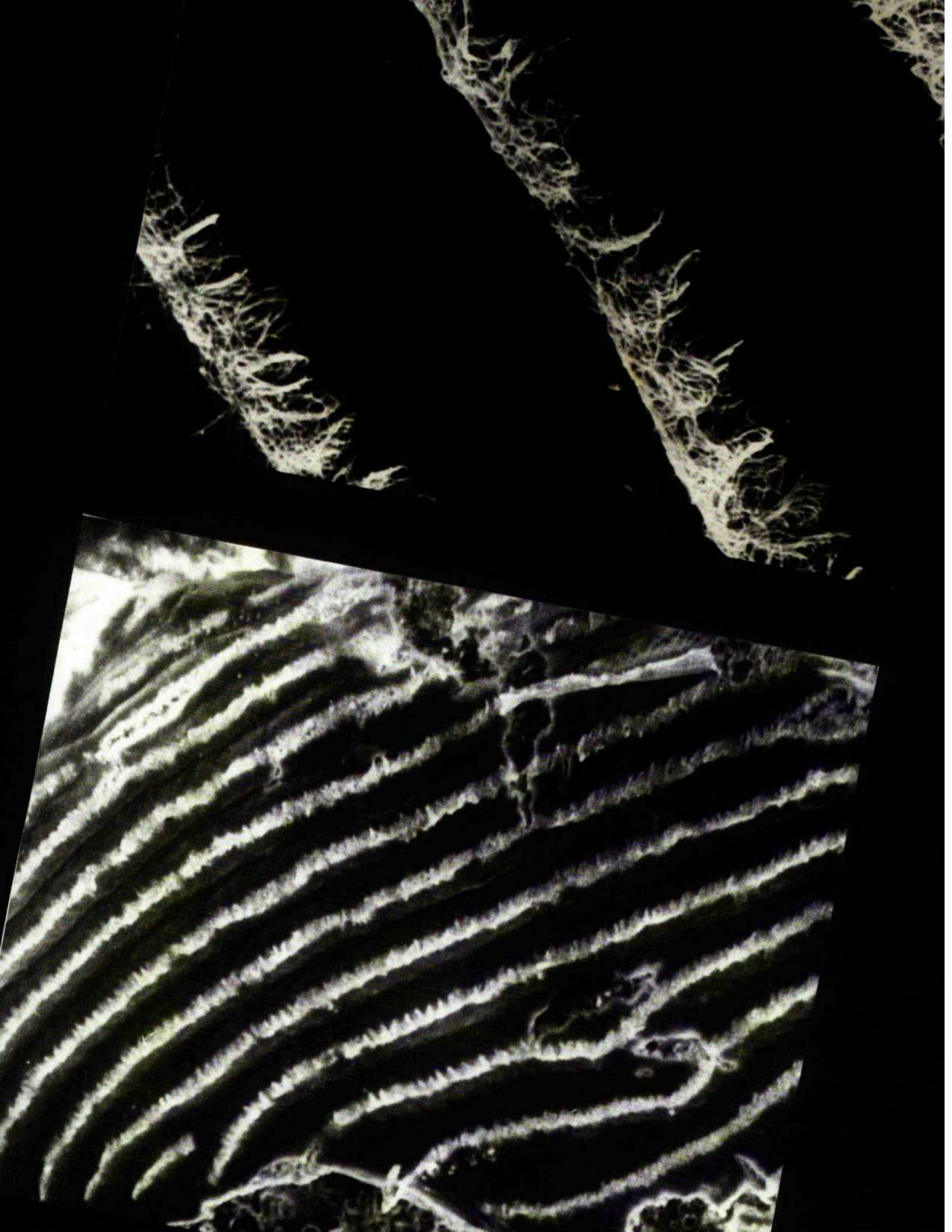


Fig. 3

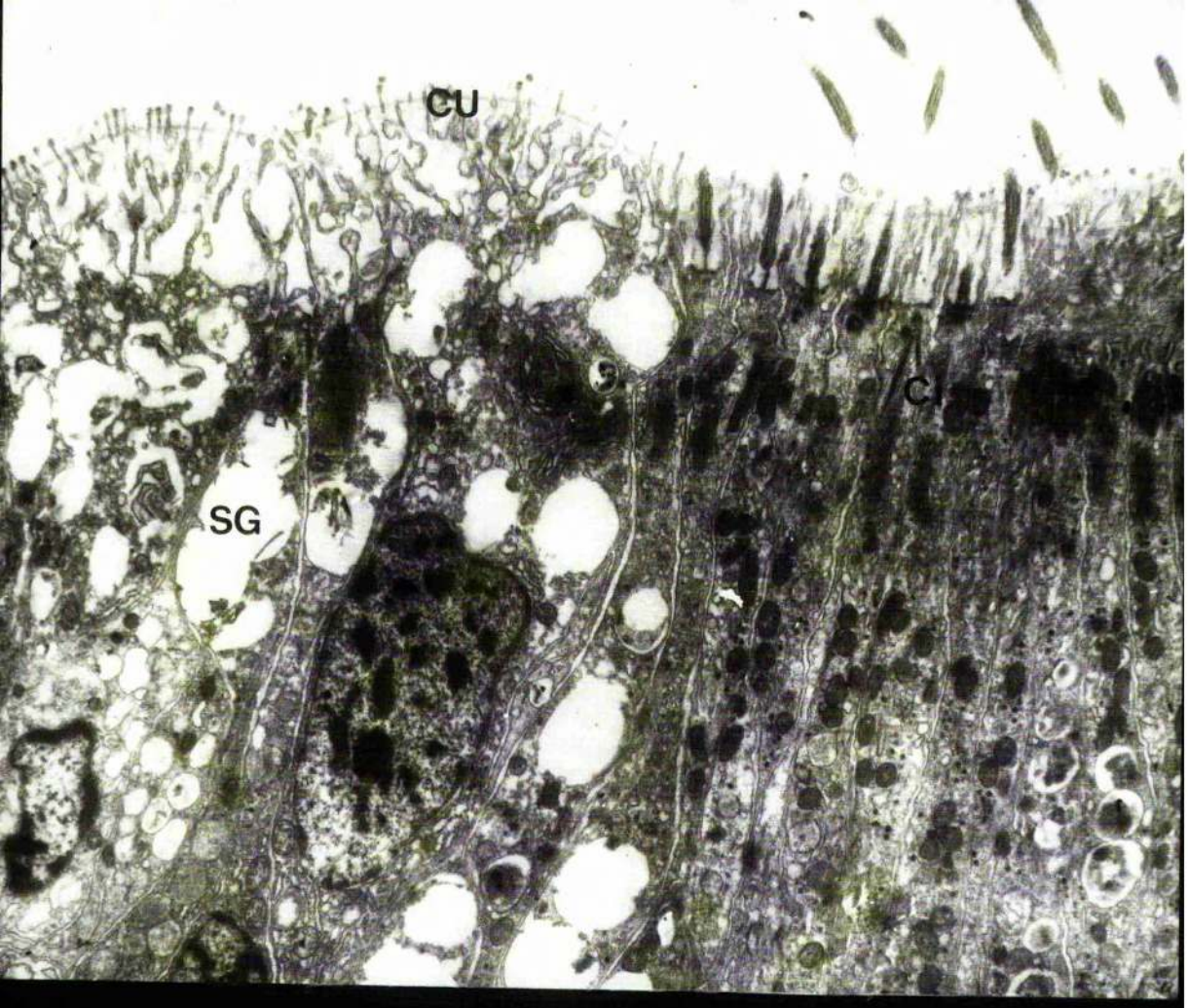
Transverse section through the edge of a ciliated ridge. Typical epithelial cells with discharged secretory vacuoles (SG) and cuticle(CU) are present adjacent to the large number of small elongate ciliated cells (Ci).

x 9,000

Fig. 4

Use of a glutaraldehyde/osmium mixture for fixation as opposed to just osmium produces less vacuolation of the cytoplasm in epithelial cells and better preservation of the cuticle. A secretory cell opening to the exterior is shown (SC). The mixed fixative produces poor fixation of nervous tissue and of some of the cytoplasmic components of the ciliated cells.

x 18,000


CU
C
SG


SC

echinoderm epithelial tissue. They represent discharged secretory vacuoles and it seems likely that they have been artifactually discharged by the effect of the fixative.

Figure 5 is a longitudinal section through the ciliated cells that comprise the ridge of the genital shields. These cells are cylindrical in shape and are approximately 1 μm in diameter and 25 to 40 μm in length. The apical region of each cell bears a long cilium of a typical 9+2 structure surrounded by an extensive palisade of microvilli. Filamentous processes arise from the basal bodies of each cilium and are connected to the base of each microvillus. The structural relationship between basal bodies and microvilli in echinoderms has been previously described (Cobb,1968; Martinez,1977). Horizontal sections across the top of the cells show that the microvilli are tightly packed with the cilia protruding through them. The ciliated cells are joined at the external surface by intermediate junctions and septate desmosomes, the latter showing inter- and intra-cellular specializations (Fig.6). The junctions between the ciliated cells are typical of those found between echinoderm epithelial cells, and they are not consistent with any previously described form of electrotonic junction (See Woods and Cavey,1981). The basal body gives rise to a typical striated rootlet structure which in turn appears continuous with individual microtubules which extend towards the nucleus. A second basal body is found at right angles to the first (Fig.5). Small elongate electron-dense mitochondria are thickly packed throughout the length of the cells. A prominent feature of the ciliated cells are the Golgi bodies which are 3-5 μm in length

Fig. 5

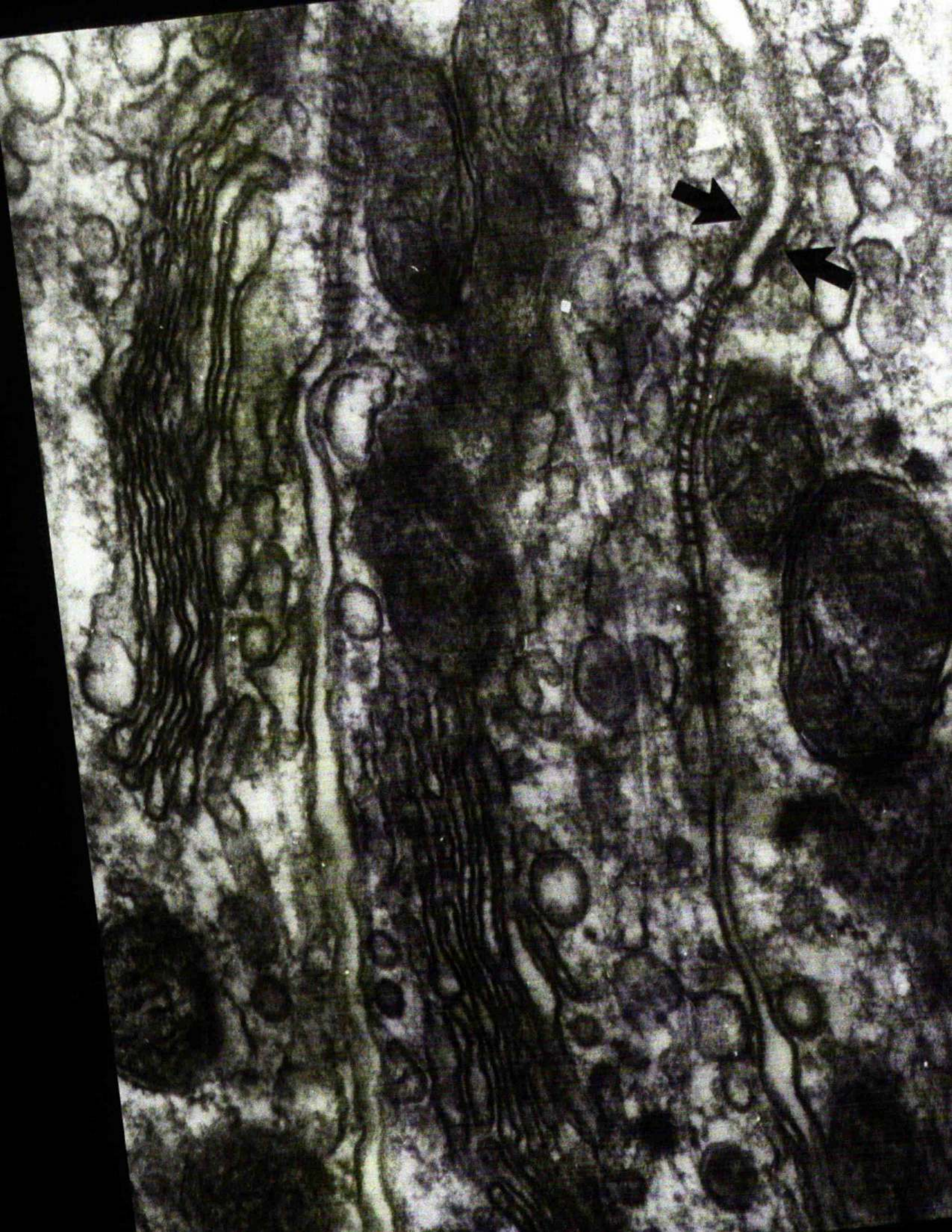
Ciliated cells cut in longitudinal section. Each cilium shows a typical 9+2 structure and basal region. Microtubules (arrow) pass down each cell from the apical region. The arrangement of the basal body, Golgi complex, secretory vacuoles, dense vesicles and coated vesicles shows clear stratification.

x 45,000

Fig. 6

The ciliated cells are joined by an intermediate junction (arrows) and immediately below this is a septate desmosome showing characteristic intra- and inter-cellular specialisations.

x 70,000



and which occupy much of the cytoplasm immediately below the ciliary rootlets. The Golgi bodies consist of 20 to 30 layers of membrane between the forming and the maturing faces and are associated with small electron-dense granules, small clear vesicles, coated vesicles and larger secretory vacuoles. Figures 7 and 8 illustrate the Golgi bodies and the associated structures.

The cell bodies of the ciliated cells are illustrated in figure 9. They occur in a layer 3 to 4 deep immediately above the connective tissue basal lamina that attaches the epithelium to the underlying calcite ossicles. The diameter of the cells in the region of the nuclei is 3 to 4 μm and is only a little larger than the diameter of the nucleus itself. Staggering the expanded nuclear regions in 3 or 4 layers allows the cells to be confined in a narrow band. Irregular cytoplasmic processes project from the ciliated cells below the nuclear region.

Many of the secretory cells which pack the epithelium of the grooves between the ciliated ridges contain extensive Golgi bodies associated with secretory vacuoles. These vacuoles are bounded by a narrow but typical membrane and contain granular material separated by filamentous processes (Figs. 10 and 11). These cells and their contents have previously been described as type B mucous cells (De Sousa Santos and Sasso,1970).

Fig. 7

Golgi complexes occur approximately 2 μm below the apex of each cell. They are associated with dense granules, clear and coated vesicles and larger secretory vacuoles.

x 55,000

Fig. 8

Secretory vacuoles (SG) are found between the Golgi bodies and the nucleus. The exact significance of the organelles associated with membrane bound secretory mechanisms in these ciliated cells is unclear.

x 55,000

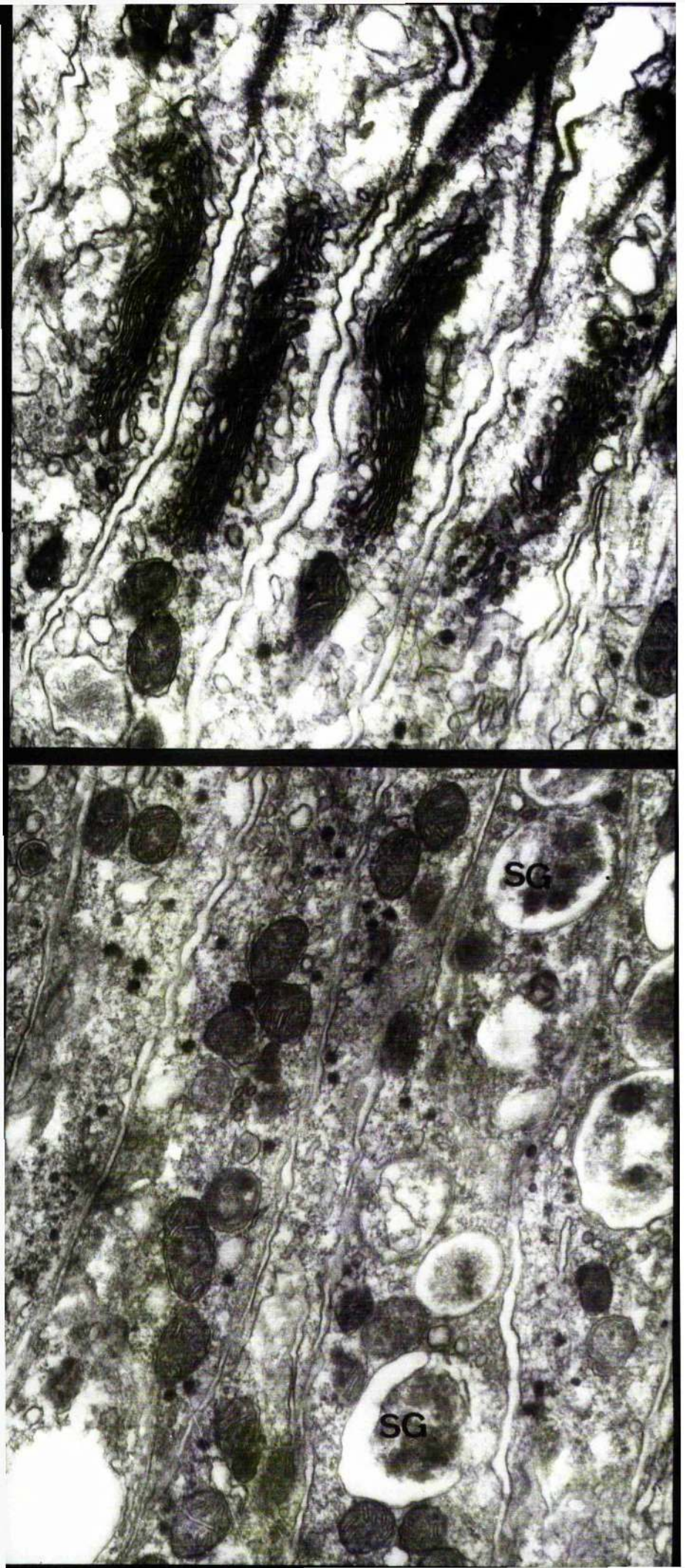

SG
SG

Fig. 9

Horizontal section through the basal region of the ciliated ridge, the narrow ciliated cells are expanded to contain a nucleus (N). The cytoplasmic processes (P) of the cell are clearly visible. Some of the nuclei are just out of the plane of section.

x 8,000

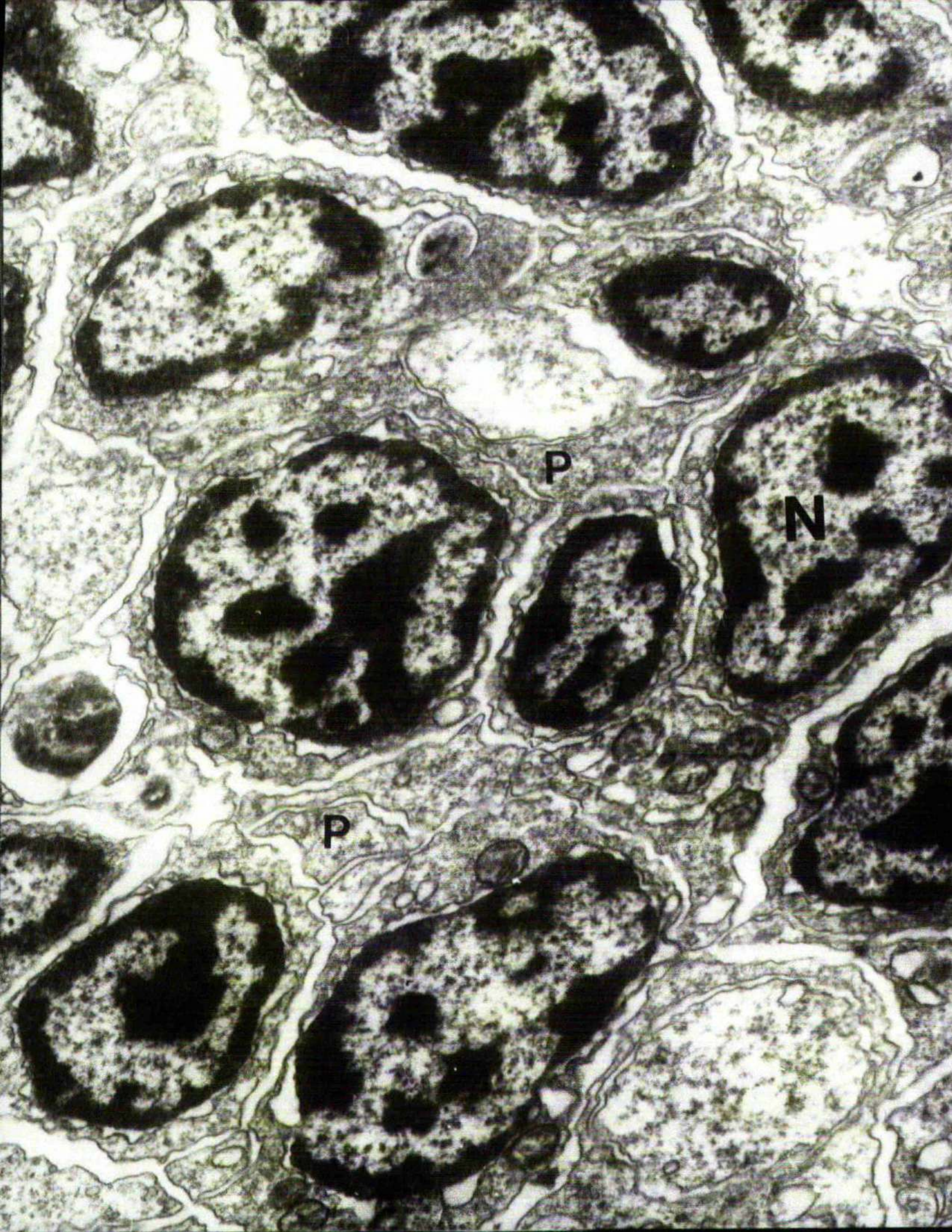

P
N
P

Fig. 10

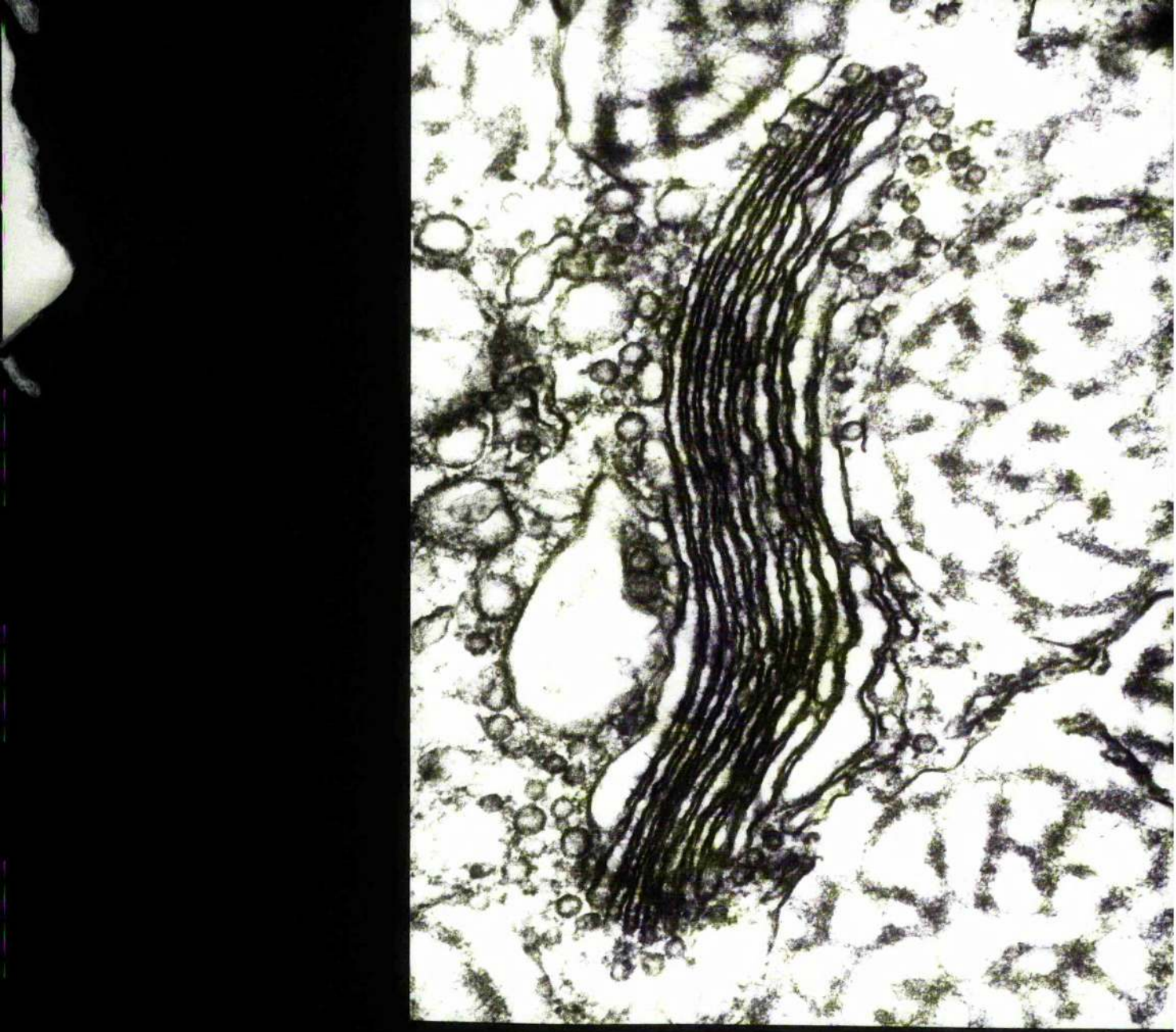
Mucous cells (type B - see De Sousa Santos,1968) occur occasionally between the ciliated cells, but are more common on either side of the ridges. The cells are packed with secretory vacuoles which are closely associated with Golgi bodies.

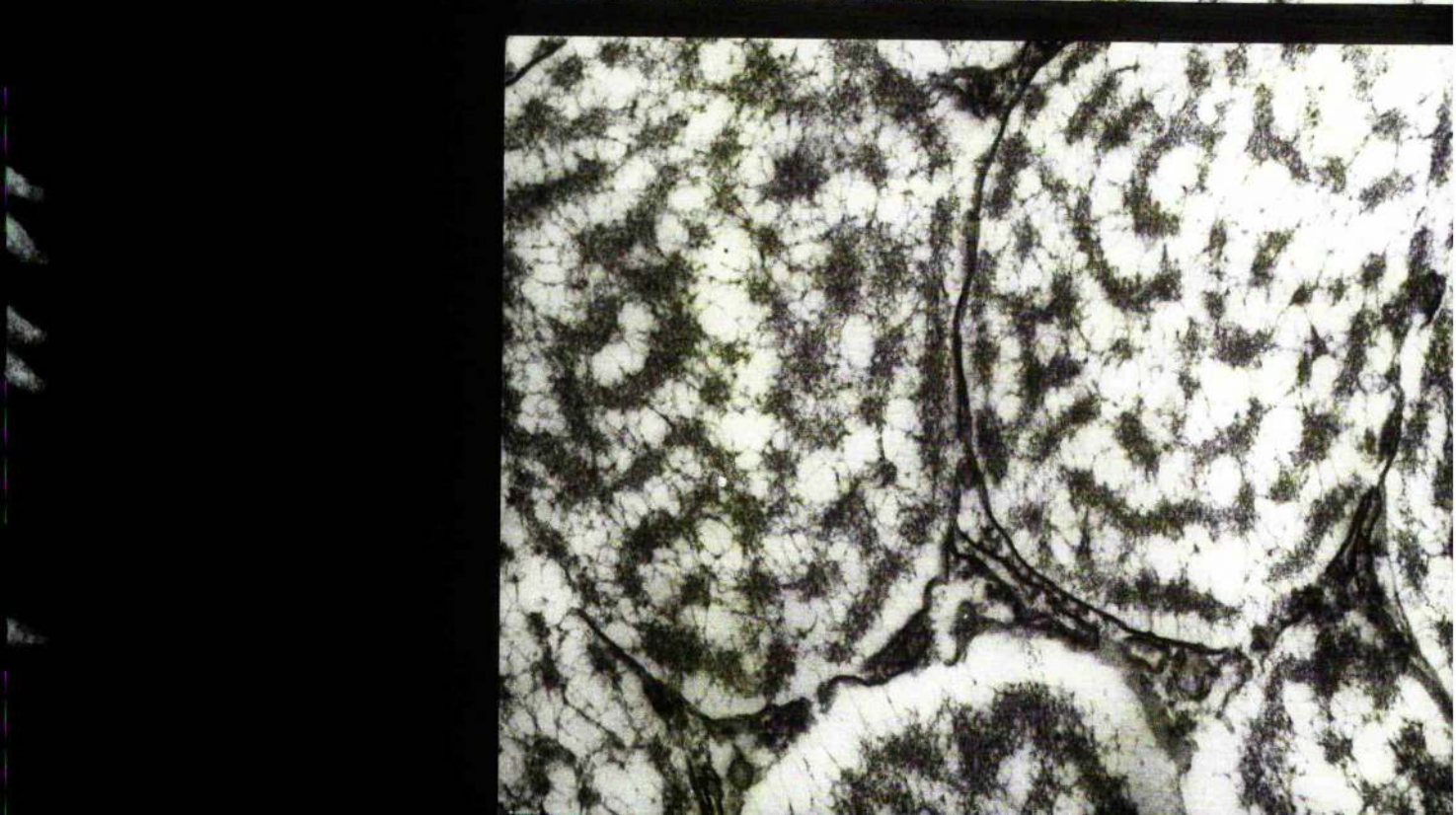
x 80,000

Fig. 11

The secretory vacuoles are membrane bound. The vacuoles contain flocculent material joined by thin filaments after fixation with osmium tetroxide.

x 40,000





Numerous small axon bundles containing between 20 and 100 fibres penetrate between the bases of the secretory cells and among the processes from the ciliated cells. Typically they contain an unstructured cytoplasm with microtubules, small agranular and granular vesicles and occasional small mitochondria. The majority of the vesicles are large granular vesicles with an electron dense core occupying the centre of the vesicle. The axon bundles break up into smaller groups and occasionally single fibres, and are clearly varicose in structure. The varicosities vary from 0.3 to 1 µm in diameter and are approximately 1 µm in length and contain mainly large granular vesicles. The inter-varicose regions of the axons are often less than 0.1µm in diameter and up to 2µm in length. They are typical of the nerve endings which occur in other situations in echinoderms (Cobb and Raymond, 1979). They form close associations with both the ciliated cells and the secretory cells but do not show the membrane or cytoplasmic structure of specialized synapses (Figs. 12 and 13).

Fig. 12

Bundles of axons (B) which are derived from the ectoneural tissue of the radial nerve cord (see Chapter 1) are irregularly arranged under the epithelium covering the genital shields. These bundles break up into groups of two or three axons and penetrate between the basal portions of of the overlying cells. They contain largely granular vesicles characterised by a lucent halo.

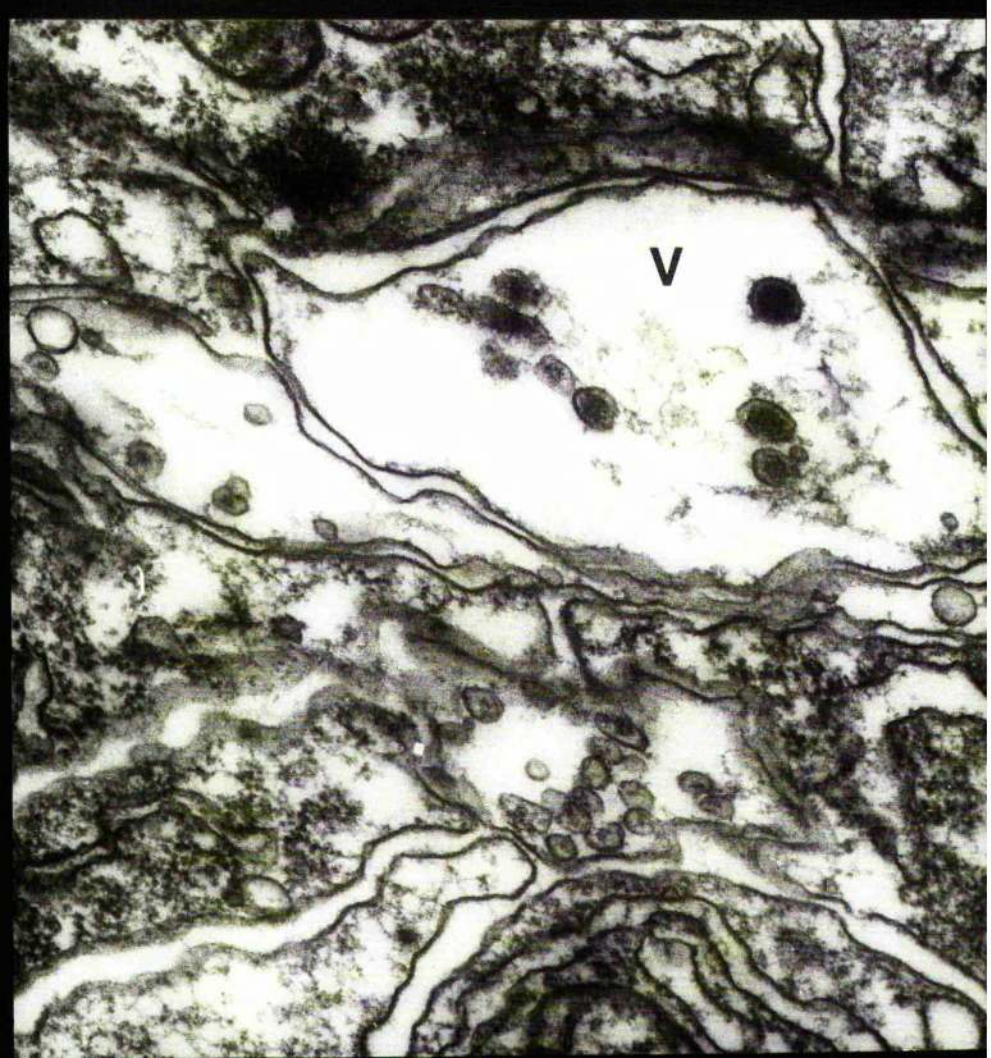
x 35,000

Fig. 13

The terminal regions of the axons are typically varicose. Vesicle-containing varicosities (V) and intervaricosities are shown.

x 60,000


B


V

DISCUSSION

Cilia are widespread in the echinoderms occurring commonly on the external epithelium, lining the gut and the coelomic cavity. They occur mainly on unspecialized epithelial cells with usually only one cilium per cell, although cells with multiple cilia have been reported (Cobb and Raymond, 1979). Cilia also occur on myo-epithelial cells (Martinez, 1976; Bachmann and Goldschmid,1978; Gardiner and Rieger,1980) and modified cilia have also been described in nerve cell bodies (see chapter 1). Specialized groups of sensory cilia have been described in the pedicellariae of echinoids (Cobb,1968) and recently in ophiuroids (Martinez, 1977a; Emson and Wilkie, in press).

The genital bursae of ophiuroids are thought to be involved in the release of gametes and in respiration. The bursa is a large cavity into which the gametes are shed and it is lined with ciliated epithelium. It occupies the inter-radial areas of the disc not taken up by the pouches of the gut and is, in places, closely apposed to the latter. Examination of other species of ophiuroid from two different families, Ophiothrix fragilis (family Ophiothricidae) and Ophiocomina nigra, (family Ophiocomidae) have shown that the complex ciliated structure found covering the genital shields in members of the Ophiuridae is not present in members of these other families, although the genital shields do bear some cilia in these other species.

The complex ciliation of the genital shields in O.texturata is involved mainly in suspension feeding. Experiments with living animals treated with carmine particles show that material drawn into the bursal slits by the ciliary currents is trapped in mucus and is then propelled out of the bursal slit towards the mouth by the action of the cilia. O.texturata is also a macro feeder and readily shows feeding activity when large food items, such as pieces of fish muscle, are proffered. The bursae and bursal slits are also involved in the release of gametes and it is possible that they have a minor role in respiration . It is unlikely that they have an important respiratory function for a number of reasons. It is improbable that Ophiura has excessive respiratory requirements that have led to the development of this complex ciliation. Ophiuroids have no effective circulatory system and little effective coelomic cavity. The bursae are well placed to provide oxygen and carbon dioxide exchange for the gonads alone. The powerful water currents created by the cilia would not be required solely for this gonadal tissue, which is present in approximately similar amounts in species not showing the specialization. The currents created by the ciliary ridges are mainly directed through the bursal slits and not into the bursal cavity.

The innervation of the genital shields by a nerve plexus containing granular vesicles provides new and important clues about the functioning of the nervous system in echinoderms. Investigations employing the fluorescence histochemical technique of Falck-Hillarp have shown a catecholamine-containing nerve plexus

underlying the ciliated ridges and grooves of the genital shields. Preliminary pharmacological experiments have shown that the ciliary beating is not affected by treatment with various transmitter substances applied to genital shield preparations at physiological and above physiological levels. Preliminary results suggest that dopamine however releases mucus from the genital shields, but further work is required to confirm this observation. Similar studies on gut preparations from asteroids, echinoids and ophiuroids have shown that ciliary beating is unaffected by the application of various transmitter substances. Dopamine also causes mucus release by these gut preparations.

The distribution of catecholamines in echinoderms has been previously investigated by use of the Falck-Hillarp technique (Cobb,1969; Pentreath and Cottrell,1970; Cobb and Raymond,1979; Huet,1981) and it has been shown that they are confined to the ectoneural nervous system. Pentreath and Cobb (1975) suggested that catecholamine-containing nerves represent a class of interneurones. Subsequently, Cobb and Raymond (1979) showed ultrastructural evidence that in some preparations there is a plexus of varicose nerve endings beneath a ciliated epithelium, and they suggested that this represented the innervation of the cilia. Preliminary pharmacological evidence now suggests that it is the release of secretory products from the cells of the epidermis and the gut that is under nervous control and not the beating of the cilia. Fontaine (1964) described in detail integumentary mucus secretion in the ophiuroid Ophiocomina nigra, and suggested that the rapidity with which mucus could be released indicated nervous

mediation. The narrow cylindrical ciliated cells of the genital shield ridges are characterised by the large Golgi apparatus and the secretory vesicles and vacuoles, and it is clear that they have retained a secretory role as well as developing a highly specialized ciliary role.

Aiello (1972) has suggested that neural control of ciliary beating is a primitive feature and that it is unusual in the vertebrates. The apparent lack of innervation of the cilia in echinoderms may well be taken as evidence of a phylogenetic relationship between this group and the chordates. Lent (1977) has recently described the innervation of mucous cells by neurones containing 5HT in the leech. Nervous control of the release of mucus has been documented in various vertebrate preparations (see Phipps, Nadel and Davis, 1980).

The presence of a variety of neurotransmitters in echinoderm tissue has been demonstrated from ultrastructural studies of vesicle types and by fluorescence histochemistry. The ultrastructural evidence produced by this study supports the view that the nerve plexus is, at least in part, catecholamine-containing. The granular vesicles present in the nerve plexus underlying the genital shields in O.texturata are similar to those described elsewhere in echinoderm nervous tissue. Fluorescence histochemical studies have shown that presence of these vesicles is coincident with the presence of catecholamines (Pentreath and Cobb, 1972 ; Cobb and Raymond, 1979 ; Huet and Franquinet, 1981). There are three basic classes of vesicles in

echinoderm nerves (see Pentreath and Cobb,1972). Small agranular vesicles (300-500 Å in diameter) which are associated with cholinergic endings. Small granular vesicles (350-500 Å in diameter) and more commonly large (700-1000 Å in diameter) granular vesicles which both have electron dense material as a central core with a lucent halo. These have been shown by the Falck-Hillarp technique to be co-incident with the occurrence of catecholamines. The vesicles in some of the axons beneath the ciliated and glandular cells of the genital shields are similar to those found in adrenergic neurones.

The structure of the cuticle, epithelium and nerve plexus in the podia of the ophiuroid, Ophiothrix fragilis, has been described in a series of papers by Martinez (1976 and 1977a,b&c). This author described modified cilia that show a 9+0 arrangement and which are borne on narrow cells among the mucous gland cells of the podia and suggested a sensory function. Some of the gland cells of the podia are also described as being ciliated. Ciliated sensory cells have previously been described in echinoderms (Cobb, 1968) but there have not been previous reports of the 9+0 arrangement. There is thus a difference between the structure of the cilia in O.texturata and O.fragilis although in both cases the cilia are thought to be involved in feeding. In the case of the former the cilia are motile and non-sensory and in the latter Martinez suggested that they are sensory but non-motile. The detailed structure of the ciliated cells in Ophiothrix clearly requires further study to confirm the sensory role of the cilia.

Suspension feeding has been previously documented in a number of species of ophiuroid (Fontaine, 1965). These species belong to the families Ophiocomidae and Ophiothricidae and they possess long arm spines. Suspension feeding involves a mucus net which is suspended between the spines and food particles trapped in the mucus are moved to the mouth by ciliary currents and by the podia. This report provides details of a different form of filter feeding which is employed by an ophiuroid that does not possess long spines, and that was previously thought not to engage in filter feeding. The genital shields of this ophiuroid are covered by an complex ciliated structure which secretes mucus in which small food particles are trapped. The latter are then wafted to the mouth by the action of the cilia.

GENERAL DISCUSSION

The study of echinoderm neurobiology has, in the past, been hindered by two major problems. The small size of echinoderm neurones has made it practically impossible to trace the pathways of individual fibres within the nerve cords, and thus to produce a detailed description of the of the fine structure of the echinoderm nervous system. The second problem has been the inability to record electrical activity from single units within the nerve cords, and this too is a direct consequence of the small size of the majority of echinoderm nerve cells. The discovery of comparatively large neurones within the nervous system of ophiuroids has allowed considerable progress to be made in the study of both the anatomy and physiology of the echinoderm nervous system.

The anatomical work described in the first chapter of this report has enabled a detailed description of the structure of the nervous system in *Ophiura texturata* to be produced. The radial nerve cords consist of a chain of peripheral ganglia connected by tracts of fibres which are aligned in parallel. Each ganglion controls the effector system of one arm segment and is connected only to the immediately adjacent ganglia; degeneration studies have shown that each segment of the nerve cord contains a discrete population of neurones.

These findings taken in conjunction with the results of the ultrastructural and physiological studies of synaptic contact between the ectoneural and hyponeural systems, across the basal lamina, confirm the basic tenets of the hypothetical model of the echinoderm nervous system proposed by Cobb (1970).

Synaptic contact between the ectoneural and hyponeural tissues occurs across the basal lamina; vesicle-filled ectoneural axon profiles are apparent in electron micrographs of the basal lamina region. Physiological evidence has been obtained which confirms that synaptic contact between the two tissues does occur across the basal lamina. Excitatory post synaptic potentials have been recorded intracellularly from hyponeural neurones in response to photic stimulation, indicating that functional contact between the purely motor hyponeural tissue and the ectoneural tissue has occurred. This lends support to the suggestions by Cobb (1970) and by Florey and Cahill (1977 and 1980) that functional synaptic contact occurs between motor neurones and the muscle layer in echinoid tube feet, across a basal lamina. Much of the recent work on synaptic function has involved freeze fracture studies of classical vertebrate synapses, but few studies of unspecialized synapses have been attempted using this technique. The area of synaptic contact between the two nervous tissues in ophiuroids provides an opportunity for the study of unspecialized synapses. The use of freeze fracture studies, would produce further detailed information about this large area of unspecialized synaptic contact, and such work would involve a quantitative analysis of the

presynaptic densities on the presynaptic fracture face, before and after stimulation of the nervous system. An increase in the numbers of presynaptic densities is taken to indicate an increased level of transmitter release.

Prior to this report the structure of the circumoral nerve ring has not been described in detail in any echinoderm. The circumoral nerve ring in O.texturata has a structure that is consistent only with providing a functional connection between adjacent radii. There are no extensive areas of neuropil within the nerve ring, and the junction region between the nerve ring and the radial nerve cords is similar in structure to the segmental ganglia of the radial nerves, with only a slight alteration in pattern to accommodate the divergence of the the circumoral nerves. Many of the results of past behavioural experiments have been interpreted in the light of the assumption that the circumoral nerve ring contains controlling centres for each of the arms, and acts as the brain of an echinoderm. The conclusions drawn from a number of past behavioural experiments will thus require re-appraisal.

The fixation of invertebrate tissue for electron microscopy is notoriously difficult (see Cobb, 1970; Cobb and Mullins, 1973; Baur and Stacey, 1977; Cobb and Pentreath, 1977; Eisenman and Alfert, 1982). Echinoderm tissue is some of the most difficult to fix adequately, and there are special problems associated in dealing with the calcified skeleton. The decalcification method devised by Dietrich and Fontaine (1975) is the most successful method yet

invented for the removal of the calcite skeleton, but even this technique results in a considerable reduction in the quality of the fixation. During the course of this study a range of fixation techniques were investigated, resulting in varying standards of preservation of different tissues; the use of gluteraldehyde as the main fixative resulted in good preservation of epithelial cells, cilia and muscle tissue, but gave rather poor results with nervous tissue, resulting in considerable distortions of the neuronal profiles. The use of osmium alone whilst giving much improved preservation of the nervous tissue, also produced a lower quality of fixation with muscle and epithelial tissue. The buffer employed in the fixative solution also affects the fixation quality; phosphate buffer was found to yield the best all round results with echinoderm tissue. The fixation technique employed for the majority of this study was aimed at producing adequate preservation of all the tissues that were studied, and was thus something of a compromise. Some success was achieved in the work described in the third chapter of this study, with the use of the fixation technique devised by Eisenman and Alfert (1982), and it would seem that for ultrastructural studies of echinoderm tissues a range of fixation methods must be employed to achieve adequate fixation of all types of tissue.

The electrophysiological studies reported in this thesis differ from previous work in a number of ways. All previous studies have dealt only with extracellularly recorded activity, and in all cases bar one, only decrementally conducted compound potentials

were recorded. These potentials have been ascribed to a summation of the activity within a large number of small units, resulting in a single compound potential (Binyon and Hasler, 1970). In all previous studies activity was only elicited by electrical stimulation of the nervous system. Extracellular recordings from the nerve cords of O.texturata failed to reveal decrementally conducted compound activity, but single unit activity could be recorded in response to a variety of stimuli. The bulk of the experiments described dealt with photically elicited responses, although responses to chemical, mechanical and electrical stimuli were also obtained. There is considerable scope for further extracellular electrophysiological work on ophiuroids, and particularly on O.texturata. The present study has not attempted to subject the extracellular data to any form of analysis other than visual analysis. Minor changes in the pattern of a response as it is conducted around the nervous system do occur; statistical analysis would yield quantitative data on the alterations in response pattern that occur. The range and intensities of stimuli to which ophiuroids are sensitive has not been fully investigated during this study, and the problems associated with electrical stimulation of the nervous system remain to be overcome.

Intracellular recordings from single echinoderm neurones have not been previously reported. Although the intracellular work described in this thesis is clearly of a preliminary nature, the results to date indicate that with certain refinements to the experimental technique, intracellular studies will yield much important information about the ophiuroid nervous system.

Particularly, it will be possible to study the role of individual ectoneural giant fibres in the conduction of responses within the nervous system, and to confirm their internunical role. It will also be possible to study the interaction between individual ectoneural and hyponeural cells, and to determine the ionic basis of the action potential. Intracellular dye-filling experiments will yield information about the detailed morphology of individual giant cells; such details cannot practically be obtained using serial section studies.

In spite of the technical difficulties associated with work on ophiuroid nervous systems, such work as well as providing further insights into the structure and function of the echinoderm nervous system, may also yield information about nervous function in a comparatively simple system. The ophiuroid nervous system has not undergone cephalization and as such the nervous elements are not concentrated into a brain, and further the nerve cords are made up of serially repeated identical units, so that information derived from the study of one segment of the nerve cord is applicable to the nervous system as a whole. The primitive position of the nervous system, close to the surface of the animal, means that the nervous system can be exposed with a minimum of dissection, leaving the animal virtually intact. The rigid form of the calcified skeleton allows only a limited range of movements to be made, so that the animal is confined to a stereotyped response to a particular stimulus.

The ciliary/mucus feeding structures that cover the genital shields in O.texturata also offer opportunities for further study. They provide a specialized preparation for the study of the function of the sub-epidermal nerves in echinoderms, particularly in relation to the control of ciliary beating and of mucus secretion.

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APPENDIX A

Fixation Methods

Baur and Stacey (1977)

1) Fix in 3% gluteraldehyde bufferred to pH 7.4 with 0.1 M Piperazine N-N-bis [2 ethanol sulphonic acid] (PIPES) for 1 hour at 22 C; continue fixation for 24-48 hours at 0-4°C.

2) Wash in 0.1 M PIPES; three changes, 20 minutes each.

3) Post-fix in 1% osmium tetroxide in 0.1M PIPES for 30 minutes.

Bachmann et al. (1980)

1) Fix in 2% osmium tetroxide in seawater for 3 hours

Coleman (1969)

1) Fix in 3% ice-cold gluteraldehyde in 0.1M sodium cacodylate at pH 7.4 for 1 hour.

2) Post-fix in 1% osmium tetroxide in veronal acetate.

Eisenman and Alfert (1982)

1) Pre-fix in 4% gluteraldehyde in 0.2M sodium cacodylate, with 0.1M sodium chloride, 0.35M sucrose and 0.05% osmium tetroxide, for 5-10 minutes at pH 7.2.

2) Main fixation stage as above but omitting the osmium tetroxide, for 1 hour.

3) Rinse in two changes (5-10 mins per change) of 0.3M sodium chloride in 0.2M sodium cacodylate (pH 7.2).

4) Post-fix in 1% osmium tetroxide in sodium cacodylate for 30 minutes.

Holland and Nealson (1978)

1) Fix for 1 hour in 3% gluteraldehyde in 0.1M cacodylate-Hcl buffer (pH 7.3) with 0.4M Nacl and 0.05% ruthenium red.

2) Rinse three times in the above solution minus the gluteraldehyde, for 10 minutes per change.

3) Post-fix for 30 minutes in 1% osmium tetroxide in 0.1M cacodylate-Hcl with 0.4M Nacl and 0.05% ruthenium red.

Hylander and Summers (1975)

1) Fix in 3% gluteraldehyde in seawater for 2 hours.

2) Wash in seawater overnight.

3) Post-fix in 2% osmium tetroxide in seawater for 1.5 hours.

Prosser and Mackie (1980)

1) Fix in 3% gluteraldehyde in 0.2M sodium cacodylate for 1 hour at room temperature.

2) Rinse in 0.2M sodium cacodylate.

3) Post-fix for 30 minutes in 1% osmium tetroxide in sodium cacodylate at 4 C.

Wood and Cavey (1981)

1) Fix in 2.5% gluteraldehyde in 0.2M Millonig's phosphate buffer (pH 7.4) with 0.14M sodium chloride; in some cases 1% tannic acid was added.

2) Rinse in 0.2M Millonig's phosphate buffer.

3) Post-fix in 2% osmium tetroxide in 1.25% sodium bicarbonate (pH 7.2) for 45 to 60 minutes.

PUBLICATIONS

Much of the work reported in this thesis has been published in the form of the following articles:

Cobb, J.L.S. and Stubbs, T.R. (1981) The giant neurone system in ophiuroids I. The general morphology of the radial nerve cords and circumoral nerve ring. Cell Tiss. Res. 219: 197-207

Stubbs, T.R. and Cobb, J.L.S. (1981) The giant neurone system in ophiuroids II. The hyponeural motor tracts. Cell Tiss. Res. 220: 373-385

Cobb, J.L.S. and Stubbs, T.R. (1982) The giant neurone system in ophiuroids III. The detailed connections of the circumoral nerve ring. Cell Tiss. Res. (In Press)

Stubbs, T.R. (1982) The neurophysiology of photosensitivity in ophiuroids. Proceedings of the 5th International Echinoderms Conference. A.A. Balkema. Rotterdam.

Stubbs, T.R. and Cobb, J.L.S. (1982) A new ciliary feeding structure in an ophiuroid echinoderm. Tissue and Cell 14(3): 573-583 (In Press).